Neural bases of navigation in foraging and play

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Abstract

Navigation is an essential cognitive skill in the life of most animals. It is moving along space in order to procure the advantages provided by different places in the environment, and to adapt to ever changing resources, dangers and needs.

Neuroscience has made great advances in understanding the neural underpinnings of navigation with the discovery of a neurons sharply tuned for spatial variables: Head Direction Cells, active when the direction the animal is facing a certain direction; Place Cells, encoding the location the animal is in; Border Cells, being active at the boundaries of an enclosure and Grid Cells firing according to the location of the animal in space, yet doing so for multiple locations organized in a perfect hexagon.

This thesis addresses the neural bases of navigation in the context of brain structure (i.e. the parasubiculum) and ethologically relevant behaviors (i.e. homing and playing).

In the first chapter I focus on the structure function relation of the parasubiculum, in the context of spatial representation. The parasubiculum is an understudied area of the parahippocampal cortex of mammals. By combining histology, tracing, identified cell recordings and extracellular recordings we performed the most comprehensive study of the parasubiculum up to date. We propose that, because of its selective connectivity with the medial entorhinal cortex, its intense internal connectivity, and the highly spatial and head directional tuning of its neurons, the parasubiculum sits in remarkable position to control grid cell activity and navigation.

In the second chapter, I study the neural bases of homing. Animals care about their homes. It is a place with a very strong positive valence associated to kin and safety. Animals are capable of returning home. We make use of the lab-rat's strong attachment to its home cage in order to study whether mammals maintain an online representation of the direction towards home, a home vector. We show, that the parasubiculum and medial entorhinal cortex do not have an explicit home vector representation, and that the presence of the animal's home did not affect global encoding of space. However, we do find

that grid cells become distorted by the presence of the home or other geometrical features affecting the internal environment.

Finally, in the third chapter, I study navigation in a complementary behavioral context, interspecies role playing game. By developing a novel behavioral paradigm in neuroscience, playing 'Hide and Seek' with rats, we are able to study mammalian play in the context of role playing and research its neural bases. We played 'Hide and Seek' with rats, and found that they acquired the game easily and played by the rules. Rats played strategically and without being conditioned developed remarkably game specific vocalizations patterns. We recorded neurons from the medial prefrontal cortex, an area associated with decision making and route planning, and found that most neurons respond sharply to different phases in the game, and may encode as well the context in which this events take place.

By emphasizing ethological approaches and free behaviors this thesis contributes to an increased understanding of the neural underpinnings of navigation in the mammalian brain.

Zusammenfassung

Für die meisten Säugetiere ist Navigation eine essentielle kognitive Fähigkeit, da der willkürliche Wechsel des Standorts es erlaubt die Vorteile eines neuen Standorts zu nutzen und somit eine Anpassung an Veränderungen von Ressourcen, Gefahren und Bedürnisse möglich wird.

Im Bereich der Neurowissenschaften gab es immense Fortschritte im Verständnis neuronaler Grundlagen von Navigation, vor allem durch die Entdeckung einzelner Nervenzellen, deren Aktivitätsmuster mit verschiedenen Variablen des Raums korrelieren: Kopfrichtungszellen sind nur aktiv wenn das Tier einer bestimmten Richtung zugewandt ist; Ortszellen feuern nur an einem bestimmten Ort im Raum; Grenzzellen werden an Rändern abgeschlossener Räume aktiviert; Gitterzellen zeigen ebenfalls ortsabhängige Aktivität, jedoch an mehreren hexagonal zueinander liegenenden Standorten.

Diese Dissertation beschäftigt sich mit der neuronalen Grundlage von Navigation im Hinblick auf Hirnstruktur (d.h. Parasubikulum) und ethologisch relevante Verhaltensweisen (d.h. Heimkehr und Spielverhalten).

Im ersten Kapitel konzentriere ich mich auf das Verhältnis von Struktur und Funktion im Parasubikulum im Kontext räumlicher Navigation. Das Parasubikulum ist ein gering erforschter Bereich des parahippocampalen Kortex von Säugetieren. Durch die Kombination von Histologie, Tracing, Ableitungen identifizierter Zellen und extrazellulären Ableitungen erstellten wir die bis heute umfassendste Studie über das Parasubikulm. Wir postulieren, dass das Parasubikulum durch seine selektive Vernetzung mit dem entorhinalen Kortex, durch seine starke interne Konnektivität, sowie wegen dem hohen Grad räumlich selektiver Aktivitätsmuster seiner Neurone im Bezug auf die Kontrolle von Gitterzellaktivität und räumlicher Navigation eine herausragende Stellung einnimmt.

Im zweiten Kapitel untersuche ich die neuronale Grundlage von Heimkehr. Tiere kümmern sich um ihr Zuhause. Es ist ein stark positiv besetzer Ort, der mit Familie und Sicherheit assoziiert ist zudem sie fast immer zurückfinden. Wir nutzen die starke Verbundenheit von Laborratten zu ihrem Zuhause um herauszufinden ob Säugetiere eine online-Repräsentation von ihrem Zuhause

besitzen, einen Heimvektor. Wir zeigen, dass das Parasubikulum und der entorhinale Kortex keinen expliziten Heimvektor besitzen und dass die Präsenz des Zuhauses keine globale Veränderung der neuralen Repräsentation des Raums hervorruft. Allerdings führte die Präsenz des Zuhauses oder anderer geometrischer Objekte zu einer Verzerrung von Gitterzellen.

Im dritten und letzten Kapitel unteruche ich Navigation im Hinblick auf Spielverhalten. Wir entwickelten ein neuartiges Verhaltensparadigma, bei dem der Experimentator mit Laborratten 'Verstecken' spielt um so ein besseres Verständnis neuronaler Grundlagen von Rollenspielverhalten zu erlangen. Ratten erlernen das Versteckspiel schnell und verhalten sich erstaunlich regelkonform. Ohne Konditionierung zeigen Ratten spielspezifische Vokalisationen. Gleichzeitige Ableitungen neuronaler Aktivität im medialen präfrontalen Kortex, einem mit Entscheidungsfindung und Routenplanung assoziierter Hirnbereich, offenbarten starke und spezifische Antworten der meisten Nervenzellen auf verschiedene Phasen des Spiels. Diese Aktivitätsmuster könnten die neuronale Repräsentation des spezifischen Spielkontextes wiederspiegeln.

Diese Arbeit liefert durch ihren ethologischen Ansatz und durch Verhaltensanalysen von sich frei verhaltenden Tieren einen wichtigen Beitrag zum besseren Verständnis neuronaler Grundlagen von Navigation im Säugetiergehirn.

Contents

Introduction

On Ethologically Relevant Neuroscience

This thesis is situated at the interphase between neuroscience and behavior, two sides of the same coin. The following introduction is intended to set the stage for the original experimental work I conducted during my doctoral studies, as well as present a philosophical approach to neuroscience that influenced this PhD.

Ethologically relevant behaviors

Nervous systems confer animals the capability to flexibly respond to changes in the environment in order to survive and reproduce. The study of the nervous system, as anything in biology, only makes senses under the light of evolution (Dobzhansky). The result of evolution on the nervous system is a remarkable panoply of species with diverse sensory systems (weakly electric fish, the star nose mole, infrared sensing snakes, and echolocating bats), motor capabilities (the cheetah, dolphins, and the peregrine falcon) and cognitive abilities (new Caledonian crows, chimpanzees, humans). Each is unique in its physiology and behavior.

Studying the behavioral repertoire of an animal requires an understanding of the world in which the animal behaves, its *Umwelt* (von Uexküll, 1934). This refers to the ecology the animal lives in both from a sensory, motor and cognitive perspectives. In a way it requires from researchers a bit of perspective taking in order to make sure that the experiments we are planning for an animal, is appropriate in the context of the world they behave in.

Not taking the Umwelt of an animal into consideration can lead to gross scientific mistakes. That is the case for example of mirror self-recognition in Asian Elephants. Mirror Self-recognition, consists on testing whether an animal can recognize itself in a mirror, implying a high level of self-awareness.

Up until the year 2006, elephants were not considered to pass this very important cognitive milestone (Povinelli, 1989), passed only by Apes, Dolphins and Humans at that point. However, new work by Fraans de Waal (Plotnik, Waal, & Reiss, 2006) showed for the first time that Asian Elephants, could use the mirror to spot a mark on their body, and that they also showed normal mirror exploration behavior. The key difference was the size and location of the mirror. Previous attempts use very small mirrors out of the reach of the animal's trunks. De Waals understanding of the elephants *Umwelt*, it's size, it's use of the trunk, resulted in an experiment with a very large mirror and the confirmation that elephants are self-aware.

Similar biases affect the study of the underlying nervous systems as well. Neuroscience has learned a lot from a reductionist approach to the brain. In vitro electrophysiology and anaesthetized in vivo electrophysiology have both helped us learn about the details of the circuitry of the brain, the molecular biology of neurons, the biophysics of excitability even the neural coding schemes for perception and action. However, studying brains in such a reductionist way that decouples physiology from behavior has its caveats.

In 1962 Hubel and Wiesel described the receptive fields in primary visual cortex in the cat, which led to their Nobel Prize (Hubel & Wiesel, 1962). They showed how robustly cells in V1 encode for oriented bars in the center of a neurons receptive field. This beautiful finding would predict that if an animal is moving its eyes naturally along a white screen with one oriented bar, then the simple cell from V1 would fire only when the fixates its eyes on the screen in the exact location that puts the oriented bar in it's receptive field. In 1996 Livingstone, did this experiment in monkeys, and found that simple cells do not spike locally as predicted but their response patters had a poor reflection of the stimuli freely observed (Livingstone, Freeman, & Hubel, 1996).

This is an excellent example to point out the importance of studying the brain under normal working conditions, under natural behavior. In a recent perspective Krakauer, points out that neuroscience has strong reductionist bias (Krakauer, Ghazanfar, Gomez-Marin, MacIver, & Poeppel, 2017). If the goal is to study how the brain generates behavior then we need to take the following into account. Supose we are trying to explain how the brain of an animal explains that animal behavior (Figure 1). We can assume the whole behavioral repertoire of an animal is a given set of behavior (Figure 1A), this whole repertoire is explained by the complete set of brain activities. If we

dethatch the study of the brain from behavior, in a dish, in a bath, under heavy anaesthesia, we must acknowledge that we are studying a set of brain activities that may not explain the behavior of that animal (Figure 1C). The suggestion made is that neuroscience needs behavior. It will be extremely relevant to understanding the brain, to actually figure out what the brain is making the animal do in the first place.

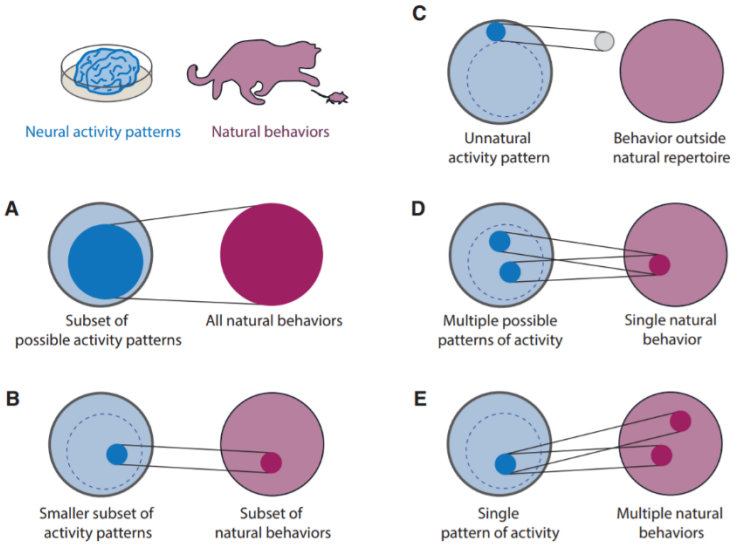

Figure 1. The Multiple Potential Mappings between Neural Activity Patterns and Natural Behaviors
(A) Of all the possible activity patterns of a brain in a dish (big pale blue circle), only a subset of these (medium dark blue circle) will be relevant in behaving animals in their natural environment (big magenta circle).
(B) Designing behavioral tasks that are ecologically valid (small magenta circle) ensures discovery of neural circuits relevant to the naturalistic behavior (small blue circle). Tasks that elicit species-typical behaviors with species-typical signals are examples (see Box 1).
(C) In order to study animal behavior in the lab, the task studied (small white circle) might be so non-ecological it elicits neural responses (small blue circle) that are never used in natural behaviors.
(D) Multiple Realizability: different patterns of activity or circuit configurations (small blue circles) can lead to the same behavior (small magenta circle).
(E) The same neural activity pattern (small blue circle) can be used in two different behaviors (two magenta circles). The circle with dashed perimeter in (B)–(E) is the subset of all possible neural activity patterns that map onto natural behaviors (from A). Figure and legend reproduced from (Krakauer et al., 2017) for clarity.

If we are convinced that the study of the brain must be linked to the study of behavior, there are also many ways of approaching the matter.
We can conceptualize all behavior used to study neuroscience under the following axes, Task Complexity, Ethological validity of stimulus response and stimulus response compatibility (Juavinett, C Erlich, & K Churchland, 2017). Under this conceptual framework (Figure 2) we can describe natural behavior as developing tasks that are both ethologically valid and have a high compatibility of stimulus response.

There is a clear philosophical difference between studying barrel cortex activity driven by whisker deflections by the presence of a pole (O'Connor et al., 2010), or study the activity of such cortex under the social behavior of face-touch in rodents (Bobrov, Wolfe, Rao, & Brecht, 2014; Lenschow & Brecht, 2015; J. Wolfe, Mende, & Brecht, 2011).
This is a very essential element to the study of the neural bases of behavior, however, most experiments conducted in the lab are mostly done far from the ecology of the animal, disconnected from the species *Umwelt* and natural behavioral repertoire.

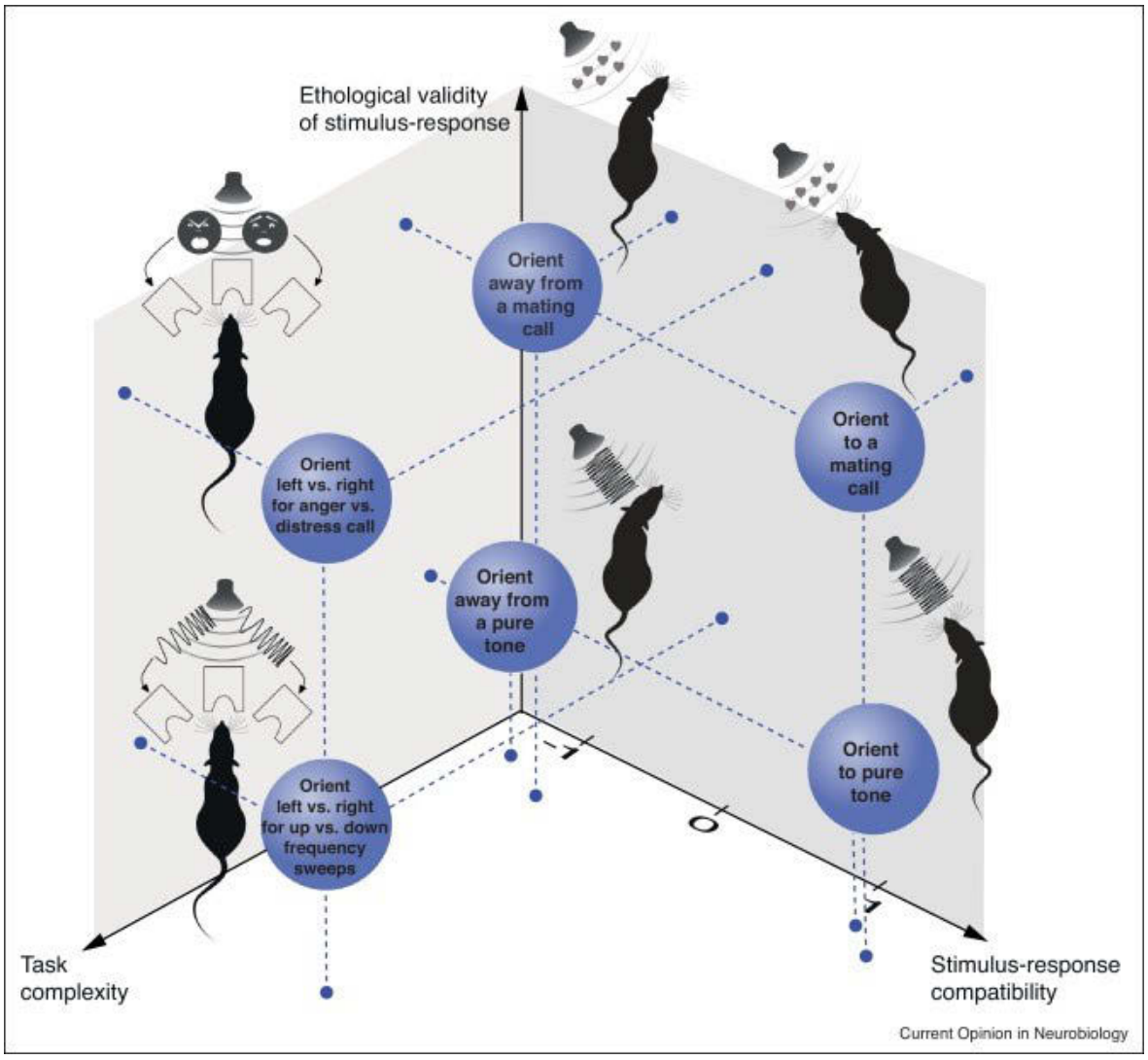

Figure 2, Axes of behavioral tasks. Extracted from (Juavinett et al., 2017)

Wireless physiology of freely moving animals

In recent years the development of technology on all fronts have prompted the occurrence of studies of neuroscience and behavior from the perspective described above. Below are a few examples of the power of recently developed techniques.

Complementing such advances in how to measure animal behavior, electrophysiology and calcium imagining have also been revolutionized by the fast growth of freely moving physiology. To date both one photon imagining with miniature microscopes (Cai et al., 2016) and extracellular electrophysiology are even possible to be conducted wireless (Deuteron tech Ltd.)

Wireless electrophysiology has allowed for the freely flying recording of spatial cells in flying bats (Sarel, Finkelstein, Las, & Ulanovsky, 2017; Yartsev & Ulanovsky, 2013) ,swimming gold fish (Vinepinsky, Donchin, & Segev, 2017) and even facilitated the recording of the electrical signatures of sleep during flight in birds flying in the wild (Rattenborg et al., 2016) .

This thesis starts with simple behavioral paradigms and increases in complexity, chapter by chapter, in part inspired by the implementation in our lab of wire-free extracellular recordings in rats. Such technical jump allowed us to study the brain under unrestricted navigation and play.

Navigation

The most basic levels of life, unicellular and multicellular microorganisms translocate to different regions of an environment deploying a variety of motor mechanisms following closely the information available about their surrounding environment. For example the chemotaxis behavior of amoeba, a single cell eukaryote.
For animals, movement in search of further information about their environment applies at many levels. This happens at the level of the senses, where animals actively sense their environment by moving their sensoriums around. This is the case with sight, requiring movement of the eyes, active touch, based on movement of limbs, fingers or vibrissae, for example.

At a more macroscopic level animals explore different parts of the global environment to forage the resources needed to survive, and to obtain information and construct memories about which regions of space have such resources in higher proportion.

Importantly, this exploration has clear homologies across the animal kingdom (Fonio, Benjamini, & Golani, 2009; Gomez-Marin et al., 2015) where similar rules seem to determine the development of spatial navigation, including the division of behavior into exploratory trips, and the increase in their complexity over time.

Notably, the more complicated the *Umwelt* and lifestyles of the animal are, their obtaining of resources of an environment sometimes includes performing rich and highly impressive translocation patterns that we refer to as animal navigation.

Different ethologically relevant behaviors require navigation in different ways. Foraging requires the extensive exploration of space in order to obtain and memorize the location of food resources, Mating and defense highlight the importance of spatial memory (Okhovat, Berrio, Wallace, Ophir, & Phelps, 2015) , both require the establishment of a territory and the gating of different behaviors when territories are breached, Hunting or Flight involves high-speed locomotion in known or unknown spatial territories, nesting and hoarding requires the transport of objects from one place to another, migration depends on large scale displacements over long periods of time.

The dissimilarity of spatial behaviors points towards the lack of a single "necessary and sufficient" algorithm, a more towards the existence of different navigational algorithms, for the effective execution of different behaviors.

Basic theories of navigation

The study of navigation is vast. As mentioned above, the concept itself encompasses a variety of behaviors and mechanistics. Navigational theory divides different behaviors in relation to the strategies used to perform them. In general, navigation is divided into the categories of beaconing, route following, path integration and cognitive map mediated.

Beaconing refers to navigation that only requires following a significant sensory landmark or gradient. This includes navigating towards a visible goal location, tracking an odor source, navigating towards a calling mate (Figure 3b, (Geva-Sagiv, Las, Yovel, & Ulanovsky, 2015)).

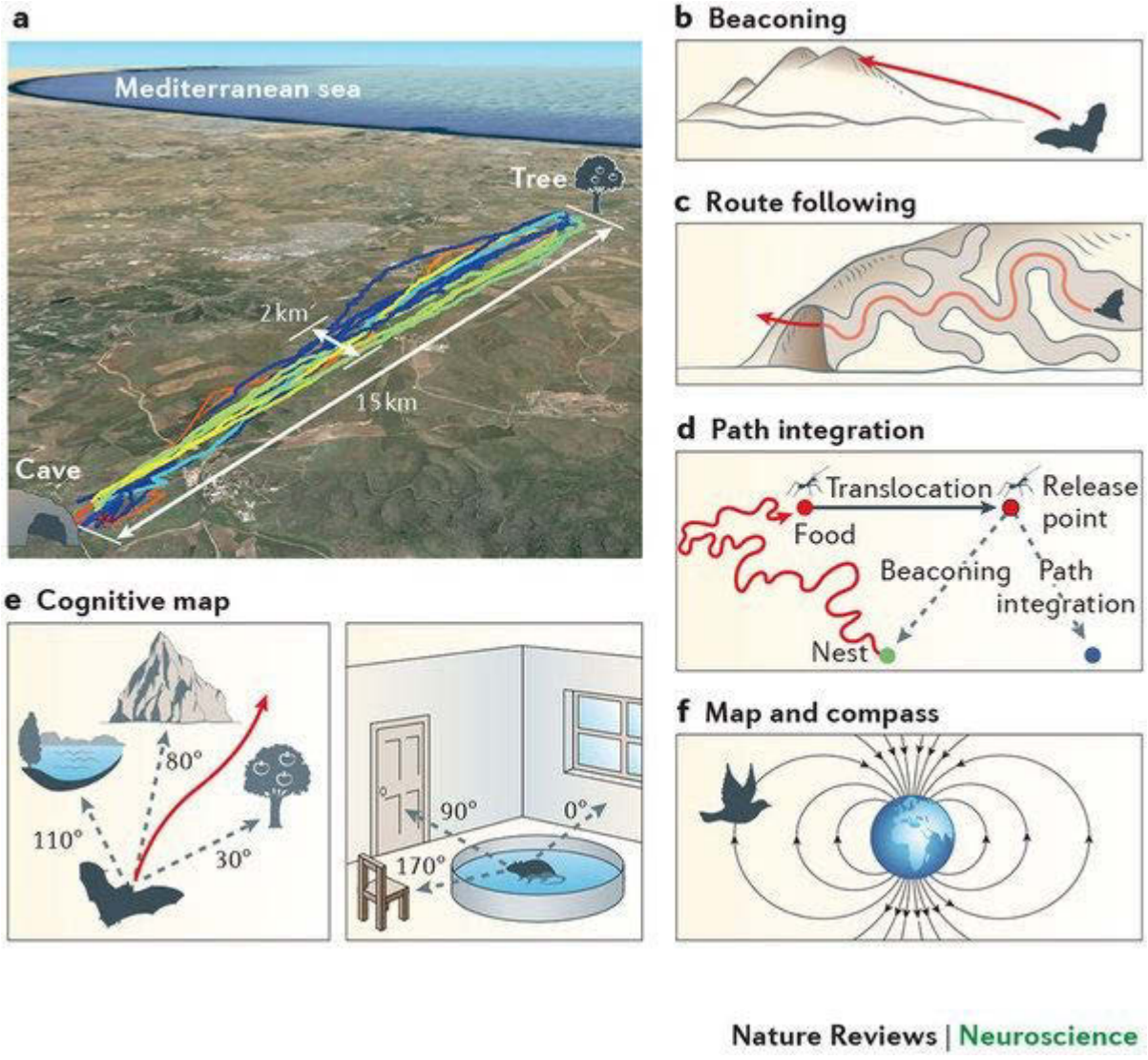

Figure 4 Different form of Navigation. Cartoon extracted from (Geva-Sagiv et al., 2015)

Route following (Figure 3c) depends on the execution of a series of maneuvers relating landmarks in the environment to specific actions. Route following does not require knowing the space, or calculating distance travelled, or absolute orientation, but depends on the execution of the right sequence of actions. A classic example would be the navigation taking place after asking for directions in a foreign city, a series of instructions to be executed at specific landmarks (street corners, crossings, semaphores).

Path Integration (or dead reckoning (Figure 3d)), refers to estimating ones position relative to an origin point by integrating past trajectory. This requires the integration of sensory information of both distance travelled and orientation. The most famous example of path integration is that of desert ants, which find their way back to the nest by computing a return to the nest vector dependent on their outwards trajectory (Wolf, 2011) . Remarkably, displacement experiments show that ants will follow the vector expected by path integration, even when translocated to a completely different place in the environment. It is very important to note that path integration is deemed to be unreliable, since the process of integration accumulates errors with the passage of time. This kind of navigation requires a frequent resetting of the integrator to be efficient.

Finally, Cognitive Map (Figure 3e, similar to Map and a Compass theory in bird ethology Figure 3f) implies a higher level of representation of the navigated space. The theory implies that the animal possesses a mental map of the environment that allows it to efficiently navigate from one place to another without requiring either the existence of a landmark at the end location or the usage of waypoints. The incorporation of path integration with landmark sensory information achieves an allocentric (external reference frame) self-triangulation which can be used to build a mental map of space.

These different navigational strategies require from the animal the use of different representations categorized clearly by the participants of the Ernts Strungman Forum 2010 (Wiener et al., 2011)in what they called the Navigational Toolbox. The Navigational Toolbox consists of 4 levels of increasing representational complexity. The first level is restricted to sensorimotor information which by itself can under certain dynamics explain all taxes and kinesis. The second level of representation pertains to spatial primitives, that is to say abstractions that can be used to perform certain behaviors, but fall short of effectively being positional information. For example velocity, a construct from integrating information from several senses can be used to implement beaconing or landing at a visible target. Velocity by itself doesn't contain allocentric spatial information, but can be combined with other second level representations to compute a position in a cognitive map (a level 3 representation). Level 3 is composed by representations of self, others, goals in an external reference frame. This is information can be used to navigate directly from two mapped locations.

Finally, the 4th level, is dedicated to spatial symbols, as was to communicate spatial information, cartography, street signs, language.
This thesis, will address the neural bases of two behaviors heavily relying on space use and understanding, the natural homing behavior and the easily learned behavior of interspecies hide and seek.

Home and Homing

Homes have a strong valence both in animals and in humans. In humans we need to look no further than the appreciation of home in our culture and vernacular, “there is no place like home”, “home is where your heart is” are a couple of examples of the importance ascribed to our homes. A home goes beyond being the roof over our heads, it becomes a place towards which we are emotionally attached.

Saving the cultural distance, this is not that different in the life of animals. Home involves different animals in very particular navigational patterns. A home is a sub-region of the total territory used by an animal characterized by the special use given to it. It is a place where certain important behaviors are better represented. Whether it is meant for resting, mating, eating, rearing of offspring, etc. The home has, in animals as well, a strong valence related to safety or social proximity.

The existence of a home or nest results in incredible navigational feats across animal species. Whether that is Sockeye Salmon returning to the stream they were born in (Neave, 1964), or albatross released far away from the nest returning to the nested island in the middle of the Pacific (Kenyon & Rice, 1958).

Homing behavior has been observed across taxa from insects (Desert Ants, Bees), reptiles (turtles (Shimada et al., 2016)), birds (pigeons (Alleva, Baldaccini, Foà, & Visalberghi, 1975)) and mammals (Papi, 1992).

Of particular interest to this thesis, homing behavior in mammals has been extensively described in many species and of two types : migrations and excursions out of the home-base (Papi, 1992) (J.Bovet, Chapter 8 Mammals, Papi book, Animal Homing.) In mammals, homing is associated to the concept of home-range, the minimal area in which mammals spend most of their time.

In general mammals spend 90% of their time in an area several orders of magnitude smaller than the area they spent the rest of their time in. These explorations beyond the home-range are called sometimes called excursions, ending in a return to their home-range, otherwise known as homing. The evidence points towards different levels of familiarity inside and outside the home-range and perhaps different processes controlling the navigation in these two areas.
In the case of homing due to migration, this consists of a mammal travelling from a seasonal home-range to another seasonal home-range they occupied the previous year. This migration repeats itself every year, with high site fidelity of the occupied home ranges demonstrated for many species of mammals, including ungulates, grey bats and marine mammals. Some mammal migrations are impressive, for example the almost straight travel of Caribou for more than 100km (Papi, 1992).
As mentioned above, non-migratory homing refers to the return from a region of space rarely visited back to the home-range or nest. Translocation from the home-range could be due to numerous factors including removal of local food resources (Rattus norvegicus,), reproductive needs like mate searching (brushed tailed possums). However, these excursions cannot always be associated to a causal reason, but are a result of exploration for exploration's sake (Baker, 1982). The function of exploration out of the home-range could be the acquisition of information about the surrounding environment.

This thesis mostly deals with homing in the context of a non-migrating animal, *Rattus Norvegicus.*

In several species of mammals, including rats. Breeding happens in a nest like structure. Wild Norway rats are a social species living in burrows where they constitute colonies (Calhoun, 1963) , build nests and cache food (Figure 4). This nesting behavior is even conserved in domesticated rats, including albino rats (Boice, 1977). This points out to the importance and the instinctive home sense of rats.

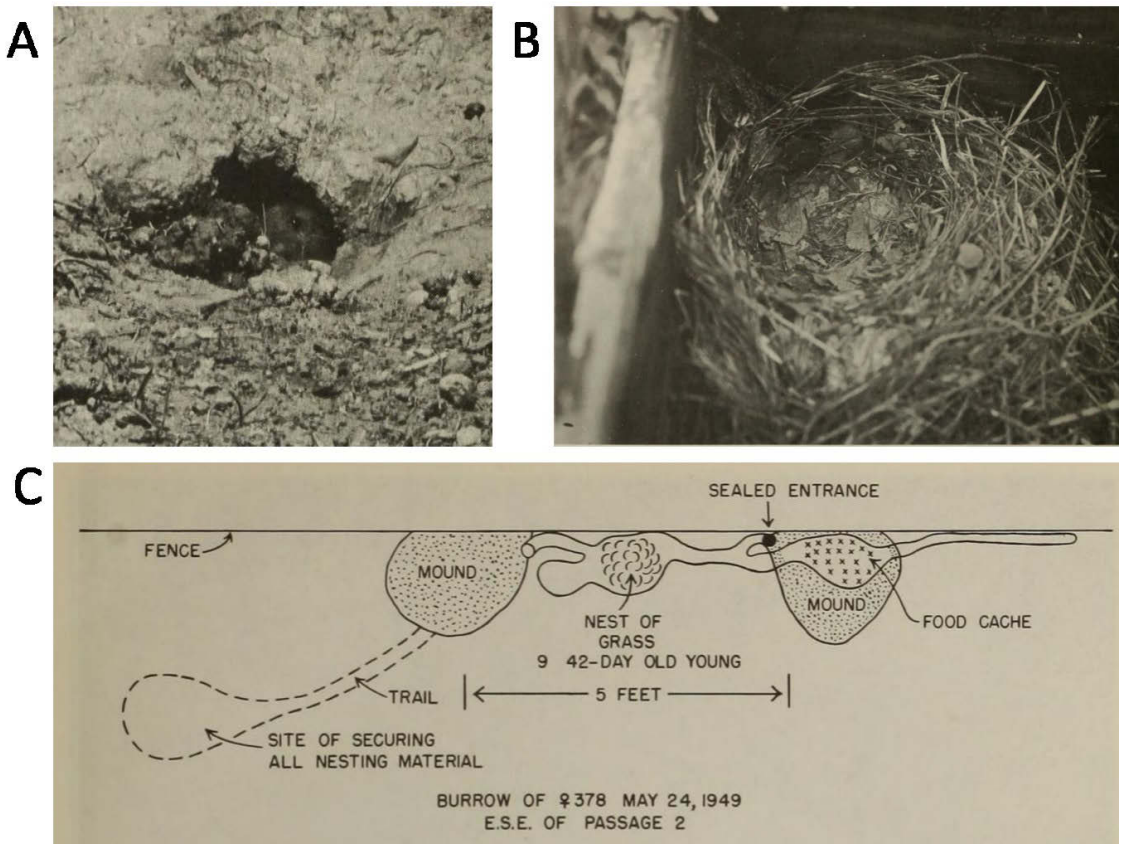

Figure 4 Burrowing and nesting of wild rats, A) Shows a Norway rat hiding inside the opening to the burrow. B) A rat nest built out of vine and leaves. C) Depiction of a developing burrow system, note the presence of a food cache and a nest. Pictures from (Calhoun, 1963).

Rat pups perform homing behavior towards their nest when displaced, or towards the smells of their nesting material(Szerzenie & Hsiao, 1977) in comparison to the nesting materials of other litters (Carr, Marasco, & Landauer, 1979). This shows a strong early valence of the home early in development.

Homing in rodents has been studied in two very specific contexts, pup retrieval **(Mittelstaedt & Mittelstaedt, 1980)** and food hoarding (J. B. Wolfe, 1939) and further developed by Whishaw through several decades of work ((Winter, Blankenship, & Mehlman, 2018.) for a recent review). Both types of studies show the capabilities of rats to orient towards their home, and perform directed, vectorial navigation.
This thesis will integrate some of the findings of homing behavior field with its neural underpinnings. Proposing a direction for future studies in the neural bases of ethologically meaningful navigation.

Navigation beyond space

One can navigate things beyond the physical space one is in. As has been mentioned above, our sensory organs move around, sampling different parts of the sensory world. For example, every time we look at a beautiful painting, our eyes move, navigating through this visual space. In fact, when humans are asked different questions about a painting their eyes will navigate the picture differently. This was beautifully illustrated by Yarbus (Yarbus, 1967) for " The Unexpected Visitors"by Illya Repin (1888) (Figure 5) .

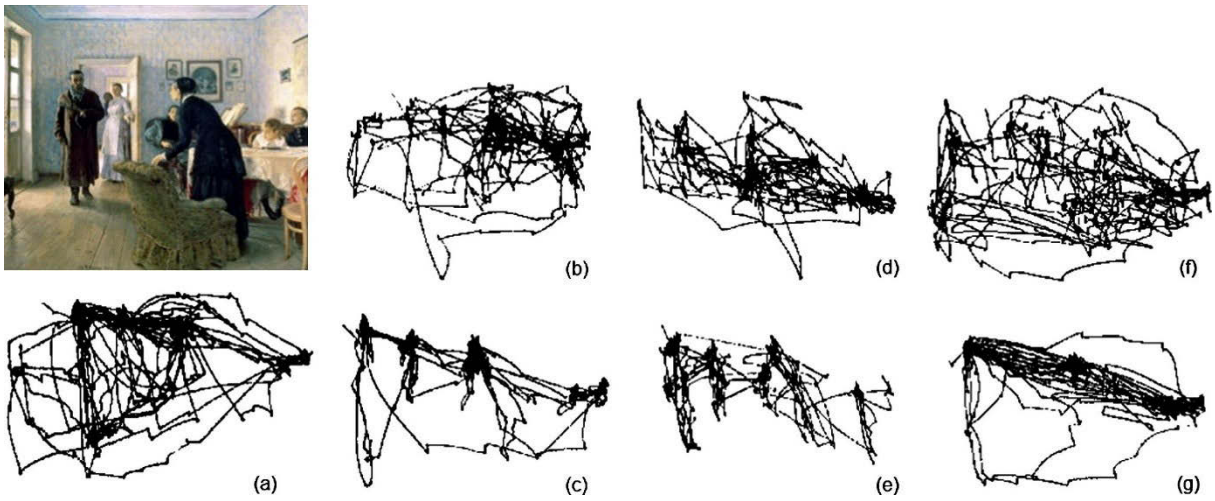

Figure 5. Eye trajectories measured by Yarbus by viewers carrying out different tasks. (a) No specific task. (b) Estimate the wealth of the family. (c) Give the ages of the people in the painting. (d) Summarize what the family had been doing before the arrival of the "unexpected visitor". (e) Remember the clothes worn by the people. (f) Remember the position of the people and objects in the room. (g) Estimate how long the "unexpected visitor" had been away from the family. Image and legend extracted from (Haji-Abolhassani & Clark, 2014).

Navigation can be extended to other domains besides space. Time, for example, can also be navigated. Humans can easilly navigate a series of events stored in declarative memory, or that are happening in real time. This allows us follow stories, movies and the life events of others. Some of the brain structures related to spatial navigation, which we will discus below, are also responsible for navigating narratives (Milivojevic, Varadinov, Grabovetsky, Collin, & Doeller, 2016).

Beyond these two inexcapable variables of space and time, humans and perhaps animals can be said to navigate other more abstract domains, like continuous conceptual spaces (Constantinescu, O'Reilly, & Behrens, 2016) or abstract relational knowledge (Garvert, Dolan, & Behrens, 2017) .

In fact, even humans complicated social networks could be navigated by the same brain structures we use to navigate space (Tavares et al., 2015). In doesn't seem farfetched that animals could also navigate their social role in a given context, or their place in a hierarchy.

Navigating Play: Hide and Seek

Play is a behavior easily recognized in humans of all cultures (Gosso, 2010) . However a clear understanding of the biological origins of play have remained elusive to science. Perhaps, the difficulty lies in the definitions of play itself. On one hand, play is defined as a free and somewhat spontaneous behavior, requiring little control external to the players. This lies in clear juxtaposition with the way most of neuroscience is conducted, mostly as highly controlled, highly stereotypical and repeatable paradigms. On the other hand, play is categorized having no immediate purpose or benefit. Again, this seems to be in contradiction with the high level of training required for most neuroscience tasks, which are based on tight reinforcement learning with either food or water deprived animals.
However most of what we know about play, comes from either from developmental psychology and the study of children at play or the study of animal behavior, where play has been described in most mammals and birds, and in reptiles (turtles)(Burghardt, Ward, & Rosscoe, 1996).
In animals play is classified in three primitive types, locomotive, object play, and social play. However these could be intermingled in more complex play pattern and can vary in their expression with development ((Gomendio, 1988) for an example of play development in Gazelles).

Locomotive play involves the early display of movement patterns that seem purpose less and resemble more an exploration of the environment and movement capabilities. Object play relates to the direct interaction with objects in the environment, again in clear lack of a specific immediate purpose other than interacting with the object itself. Finally, these leaves us with social play which requires the purposeless interaction with other conspecifics or even animals of a different species. As might become evident to the reader, these 3 types of play seem to follow a pattern of increasing complexity in the type of interaction required. With this perspective, play could be seen as behavior related to the simulation and development of ways of interacting

and testing the affordances of the environment, its objects and its living beings. Play, seen as behavioral experimentation, may contribute to the development of new potentially beneficial behaviors in a species, and thereby be selected in evolution (Fagen, 1974).
In rats, an insightful finding in the study of play has been the fact that several positive and rewarding behaviors elicit vocalizations (Burgdorf et al., 2008). Vocalizations of 50kHz are frequently associated to positively valence behaviors like mating and play. In experiments of self-administer playback of recorded vocalizations, rats preferred to replay 50 kHz vocalization in contrast to 22kHz vocalizations (a frequency associated with negative valence situations).
Further on, rat 50 khz vocalizations also produced during heterospecific play, in the form of tickling (Panksepp & Burgdorf, 2003), suggesting these might be an evolutionary precursor of human laughter. The fact that such positive valence and play like interaction is possible from interspecies interaction should not surprise any pet owner. The involvement of an experimenter in the interaction on the other hand have allowed for a certain level of control of the play, this has been critical for further scientific decomposition of this most remarkable response, and the dissection of the neural circuitry responsible (Ishiyama & Brecht, 2016).

Inspired by the possibility of controlling the behavior of the human at play with a rat we were interested in studying other more complex forms of play. Interestingly, none of these forms of play need to be „invented “, since they are already part of our game corpus, and many of them involve spatial navigation.

Let us think of a dog. What does a human play with a dog? Fetch comes to mind, a spatial game, where the human throws an object far away, and the dog finds it, retrieves it, and the game commences again. Dogs are also capable of finding hidden objects as a form of play. Both these games require a use and understanding of space. Another game that has recently caught on with interspecies play, is the well-known human game of Hide and Seek.
Hide and Seek is a children's game, possibly described first by 2nd century greek writer Julius Pollux, and played in different variants by different cultures under different names, escondite in Spain, jeu de cache-cache in France, de-av-ati ascunselea in Romania , machboim in Israel, sumbaggoggil in South Korea, escondidas (Ecuador, Uruguay, Argentina and Chile),

and cucumbè (Honduras and El Salvador) as a few examples (from encyclopaedia Britannica Online).

Hide and Seek involves several players divided in two roles, The Seeker (1 player) and the Hiders (>1 players). At the beginning of the game, the Seeker gives a certain amount of time for the rest of the players to hide themselves from view in a certain space. Hiders, must disperse and try to effectively find regions of space where they can avoid being seen or heard. After the allocated time, the Seeker announces it starts seeking, and announces every time somebody has been found. Usually, the first Hider to be found will take the role of the Seeker in the second round.
Hide and seek, seems like a very interesting game requiring many cognitive capabilities, like spatial navigation and spatial comprehension, strategy and even theory of mind. Beyond the navigation of physical space, Hide and Seek requires navigation of a game structure, the narrative of a game, as well as navigating the role one has in the game. All these properties make Hide and Seek a very interesting topic of study. However, Hide and Seek has barely been studied by academics, perhaps because of its origin as a child's play.

Neural basis of navigation

Playing a game like Hide and Seek requires understanding space and navigation from one place to another. Navigation as a higher level cognitive function that integrates information from different senses as well as motor activity. As has been described in previous sections, different forms of navigation require different types of information

Important work by Tolman in the 40's pointed towards rats having a memory of places. In a set of classic maze experiments Tolman trained rats to find a reward in a simple maze in location G (Figure 6). After training rats started navigating from the same origin, but now found their route to the reward blocked, and many possible arms to follow. Using the starburst maze Tolman showed rats would preferentially take the arm of the maze that led in the direction of the reward implying a memory not of the route but of the space

and the location of the reward (Tolman, Ritchie, & Kalish, 1946). This finding and others led him to stipulate the existence of a cognitive map in rats and men (Tolman, Cognitive maps in Rats and men, 1948)

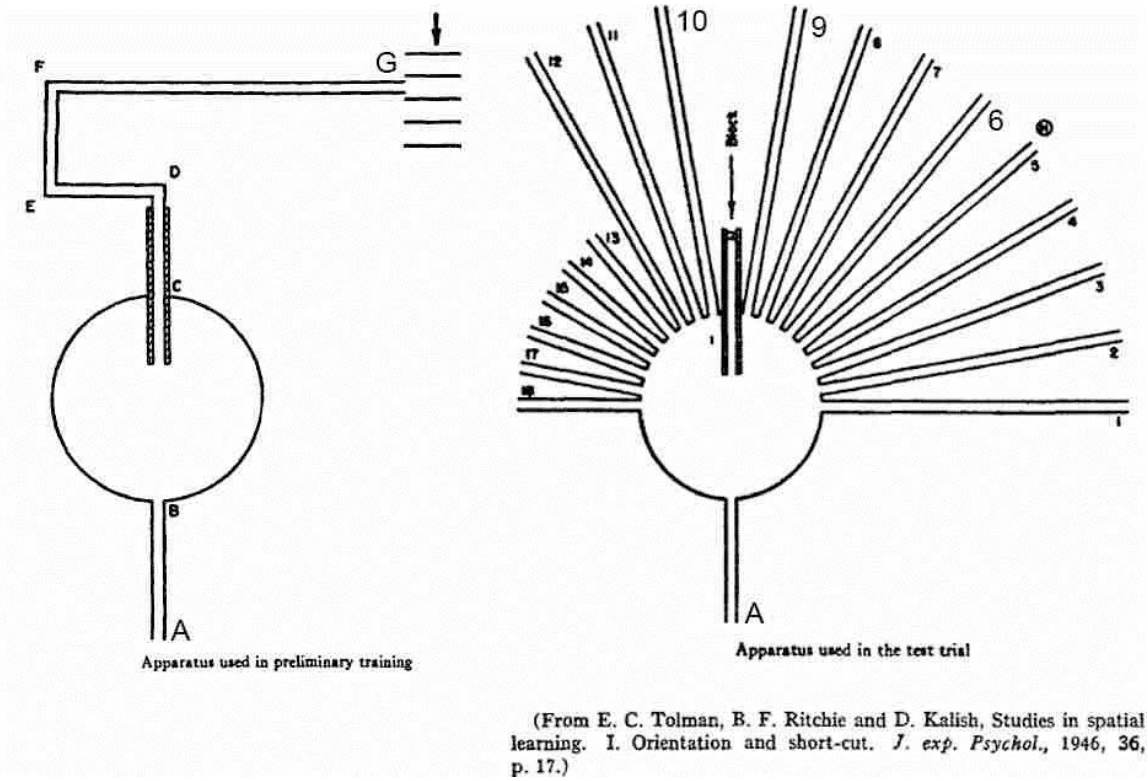

Figure 6, Tolman's maze, Left: Preliminary training apparatus with the goal location demarcated by the letter G. Right: Test apparatus forcing the rat to choose a different arm and estimate the one that leads to the learned location (H). (Originally in Tolman, Ritchie, and Kalish 1946)

Tolman's idea would find its neural bases in the 70's when work by O'keefe and Nadel conceptualized "The Hippocampus as a Cognitive Map" (John O'Keefe & Nadel, 1978). After an extensive review of the disparate literature on this most remarkable structure, and the discovery of the place cell in the hippocampus in 1971 gave birth to a whole new field in neuroscience that would lead many decades later to the discovery of a myriad of cells, and neural structures that allow for navigation in mammals. Culminating in 2014, when the Nobel Prize for Physiology and Medicine awarded to O'keefe, Edvard Moser and May-Britt Moser.

Here I will briefly review some of the cells and structures that are encompassed by this thesis. Place cells (Figure 7A), cells that produce action potentials only in a certain region of the environment. Head Direction cells (HD cells), which are highly tuned to the direction in which the animal is looking. Grid cells, which encode space with multiple fields of spiking activity

organized in a hexagonal lattice that covers space, and Border cells, active only in the boundaries of an environment.

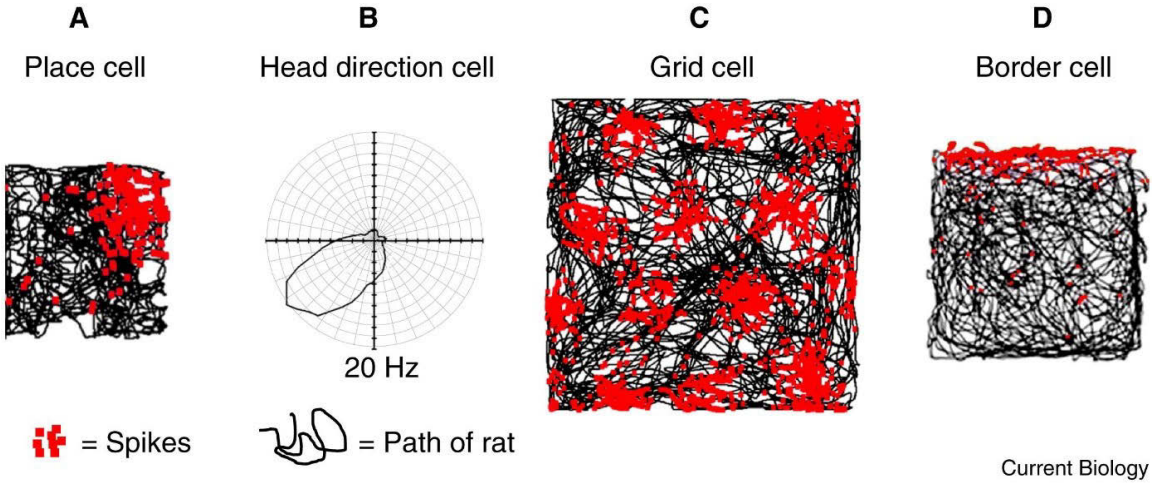

Figure 7, Cellular bases of space and navigation. A) Depiction of a Place cell. Red squares represent the location the rat was in when the place cell spiked. Black lines represent the trajectory of the animal. This shows how the cell is active exclusively in a region of space. B) Head direction cell. This circular plot shows the angular tuning curve of this cell to the head direction of the animal. C) Grid cell. Show in the same way as A, this figure demonstrates the hexagonal arrangement of Grid cell spiking activity. D) Border Cell: Spikes for this functional cell type only occur at the boundary of the environment. Figure from (Marozzi & Jeffery, 2012)

Place Cells

The introduction of recording in freely moving animals allowed for the first time the study of brains of animals navigating in space. In 1971, O'Keefe and Dostrovsky published their seminal paper describing cells in the hippocampus of freely moving rats showing a spatial receptive field (O'Keefe & Dostrovsky, 1971). Further work (O'Keefe, 1976) showed that different cells in dorsal hippocampus where active in different regions of the explored environment, different place fields. The concept of place field extended that of the receptive field from purely perceptual into a cognitive realm, since the activity of the cells was not explained unequivocally by what the animal was seeing, smelling or touching, as a series of experiments definitely proved that place cells were stable in the dark (Quirk, Muller, & Kubie, 1990), did not change firing in space dependent on the direction the animal was looking (Muller, Bostock, Taube, & Kubie, 1994). This work established for the first time a cellular basis for a cognitive function.

The discovery of cells selectively active in places in the environment and Tolman's work on the capability of rats for remembering places inspired

O'Keefe and Nadel to propose the hippocampus as the neural substrate for a cognitive map. And the place cell as its cellular building block.
After the technical development of steretrodes and tetrodes, simultaneous recordings of large populations of place cells showed that indeed the activity of different cells maps the entire environment (Wilson & McNaughton, 1993). This implies that a population of place cells would be able to encode the position of the animal in space, giving further confirmation to the cognitive map hypothesis.
Complementing the finding that place cells encoded completely an environment, a series of studies showed that place cells remapped for different environments (Muller & Kubie, 1987). That is to say that individual cells altered the position of their place field or stopped having a place field at all in a different environment. This implies that the hippocampus builds different cognitive maps for different environments, an interesting corollary for such a development is that if different populations of cells are recruited differentially in different environments, then population wise the hippocampus is also capable of encoding the identity of the environment itself (Rich, Liaw, & Lee, 2014).
Decades of work would show, that beyond this correlation between the activity of hippocampal cells and the location of the animal in space, place cells and the hippocampus itself might be involved in the planning of navigation and memory of places and routes. Preparatory (pre-play) activity of sequences of place cells related to future trajectories the animal will take would suggest a role for place cells in planning future movements though the environment (Pfeiffer & Foster, 2013). A clear causal role, for place cells in spatial navigation has mostly eluded us. However, recent studies are striving to demonstrate this causal role using very clever experimental design. Closed loop stimulation of reward circuitry driven by single place cell activity during sleep resulted in the animal exploring preferentially the rewarded area of space (de Lavilléon, Lacroix, Rondi-Reig, & Benchenane, 2015).

The phenomenology of place cells and other cells to be described is incredibly extended, and has been reduced to bare bones to facilitate the reading of this thesis.

Head Direction Cells

Head Direction Cells (HD cells) are cells showing a strong preferential tuning of their activity in relation to the cardinal orientation of the head of the animal. In head direction space, these cells fire selectively when the animal is looking in a certain alocentric direction irrespective of where the animal is located in space (FIGURE HD cell). These cells discovered by Ranck and Taube were first described in the postsubicullum(Jeffrey S. Taube, Muller, & Ranck, 1990b, 1990a) and subsequently have been found in a variety of brain regions that extend from the lateral mammillary nucleus, the Anterodorsal Nucleus of the thalamus (Jeffrey S. Taube, 1995) into the hippocampal formation, CA1 (Leutgeb, Ragozzino, & Mizumori, 2000), Presubiculum, Pasasubiculum (Boccara et al., 2010) , Medial Entorhinal cortex (Sargolini, 2006), Retrosplenial cortex (Jacob et al., 2017), postrhinal cortex (Kornienko, Latuske, Bassler, Kohler, & Allen, 2018).

The circuit for the head direction system, has been extensively described (Taube Review). One of the most critical findings into the origin of head direction cells is that vestibular function is required for head directional activity in the anterodorsal nucleus of the thalamus (ADN) (R. W. Stackman & Taube, 1997) and the Presubiculum (Robert W. Stackman, Clark, & Taube, 2002).
Vestibular information in the form of angular head velocity information encoded by the Dorsal Tegmental Nucleus is integrated into a circuitry involving the lateral mammillary nucleus and the ADN where the first head directional activity is found (Jeffrey S. Taube, 1995).

In a similar way to how place cells are encoding for different regions of space, each HD cell in the system is tuned to a single preferred allocentric head direction. However, the population of head direction cells represents all possible allocentric directions as described for example for all layers of medial entorhinal cortex (MEC) (Giocomo et al., 2014).

One of the most interesting properties of Head direction is how their orientations are defined in relation to landmarks in the environment. Firstly, it's important to mention that head directionality, is somewhat stable in darkness (Goodridge, Dudchenko, Worboys, Golob, & Taube, 1998). Visual information is not needed to produce or maintain the head directional signal, which has a persistent quality (J. S. Taube, 2003). However it is important to maintain a stable representation that avoids drift (Goodridge et al., 1998).

Sensory cues, which include visual cues, would help anchor the head direction system.

Visual cues have been by far the most studied. Early experiments by Taube (Taube et al, 1990, 1995) showed that in simple environments with a single important visual cue (such as a white cue card on a dark cylindrical environment) HD cells would align their activity to this central cue. Rotation of the cue in the reference frame of the environment resulted in a rotation of the preferred head direction of the cell by the same angle.
There are two important things to note about these experiments, which will contrast with the results of this thesis. Cue rotation experiments involved the landmark being rotated out of the view of the animal and the animal was usually disoriented before being returned to the environment. If the animal is in view of such rotation only few head direction cells rotate and they do so in a less accurate fashion (Taube 1990b).
Working in more realistic environments HD cells have been reported to realign themselves more effectively to distal background cues than to foreground visual cues (Zugaro, Berthoz, & Wiener, 2001).

Even though these remarkable findings point to head direction cells as a compass in the brain, only few studies have shown their relation to navigation. A clever use of a path integration task in rats, showed that the error of a return trip to a starting position (in the darkness) correlates with the shift in the head directional activity during the return. Implying that errors in path integration could be due to uncompensated errors in the head direction system (Valerio & Taube, 2012).

Border Cells

Vector Boundary cells or border cells, are spatial neurons with preferential firing along a boundary or wall of an environment. Such cells were first predicted to exist by O'keefe and Burgess in the 90's (Hartley, Burgess, Lever, Cacucci, & O'Keefe, 2000; O'Keefe & Burgess, 1996) in order to explain the elongation of place cells due to stretching of an environment (O'Keefe and Burgess, 1996). The existence of such cells was confirmed for the subiculum by Barry in 2006 (Barry et al., 2006) (expanded by (Lever, Burton, Jeewajee, O'Keefe, & Burgess, 2009)) and for the medial entorhinal cortex by the Moser group in 2008 (Solstad, Boccara, Kropff, Moser, & Moser, 2008). Since then

border cells have also been found in the presubiculum and parasubiculum (Boccara et al., 2010).
The main properties of border cells include environment independence. Border cells show similar activity in different environmental configurations (Solstad et al., 2008). Interestingly, cells also show repeatability of their firing field. The inclusion of new walls with the same orientation as the preferred wall, result in the appearance of a new firing field (Lever et al, 2009).
Importantly, border cells in the subiculum do not only respond exclusively to boundaries of the environment delimited by walls, but also show firing fields next to drops (Stewart, Jeewajee, Wills, Burgess, & Lever, 2013) .
One of the most interesting roles suggested for this type of cell is related to the resetting of path integration. Clearly, being close to a border in an environment, gives important information about location. This way boundaries may play a role as an error correcting signal for grid cells (Hardcastle, Ganguli, & Giocomo, 2015). This effect could be mediated by border cells containing such information.

Grid Cells

In the search for spatial representations upstream of the hippocampus, the Moser group studied the Medial Entorhinal Cortex, one of its main inputs, discovered a new functional cell (Fyhn, Molden, Witter, Moser, & Moser, 2004) with multi-peak spatial place fields arranged in an hexagonal lattice. The denominated this cell type, Grid cell (Hafting, Fyhn, Molden, Moser, & Moser, 2005). Grid cells were describes as tessellating the whole environment with firing fields arranged in a grid composed of triangles or hexagons. This representations were described as stable for a given environment in independent trials (Fyhn et al., 2004), and having consistent properties across environments of different sizes and shapes (Hafting, 2005) and conserving their activity under darkness (Hafting et al., 2005).

Grid cells have three main properties: orientation, the direction of the hexagonal grid; spacing, the distance between neighboring firing fields, and phase, the location of the nodes. Even though grid cells recorded by the same electrode showed unrelated phases, the orientation and spacing of neighboring grids is similar (Hafting et al., 2005). Further on, it was found that grid cells in the MEC are organized in discrete modules in terms of orientation

and spacing (Stensola et al., 2012). Spacing of grid cells increases along the dorsoventral axis of the MEC (Stensola et al., 2012).

Some grid cells have been found to conjunctively encode the head direction) as well as the speed of the animal (Sargolini, 2006).

Grid cells were originally found in layer II of the MEC, but their finding was extended to layer III and deep layers, as well as onto other parahippocampal areas, the pre and parasubiculum (Boccara et al., 2010).

Some of this anatomy/function correlates have not been without controversy and are still in need of a consensus. There is no other paper describing grid cells in the Parasubiculum and presubiculum, besides very early work by Caccuci who shows “place by direction” cells, which seem to be in many cases multi-peaked and in some cases just very clear grid cells (Cacucci, 2004). The finding of grid cells in layer III could not be confirmed using anatomically identified juxtacellular recordings in a study led by our group, which I coauthored (Tang et al., 2015).

The presence of grid cells in layer II of MEC, is without dispute. However, work by our group on the anatomical identity of neurons in layer II revealed that the two main cell types, pyramids and stellates have remarkably different anatomical organization. Pyramids are organized in hexagonal patches across layer 2 of MEC (Ray et al., 2014). This very suggestive finding pointed towards an important role of pyramidal cells in grid cell population, which went clearly against the predominant idea that stellate cells, because of their resonant properties in slice, might be the ideal candidate for being the grid cells. The work of our group, using juxtacellular and tetrode recordings showed a result to the contrary establishing that pyramids are grid cells as well (Tang et al., 2014). More recent work has shown a more balanced picture, pointing towards a similar percentage of grid cells in both populations (Rowland et al., 2018; Sun et al., 2015).

Even though grid cells have been found to be remarkably stable in their pattern of activity (Diehl, Hon, Leutgeb, & Leutgeb, 2017), several studies show how grid cells can reconfigure themselves in several ways. Initially, it was found that in similar fashion to HD cels, grid cells follow landmark cue rotation (Hafting et al., 2005), perhaps due to the disorientation of the rat. However

both Diehl and recent work has shown incredible robustness of grid cell node locations across different environments, but at the same time described how firing fields may change their rates in order to encode for a different location (Diehl et al., 2017; Ismakov, Barak, Jeffery, & Derdikman, 2017).

The global gridness of cells, i.e. the hexagonal position of the nodes, seems to only be affected by geometry of the environment, either in the presence of a hairpin maze (Derdikman et al., 2009), which disrupts completely the grid pattern or by the irregularity in shape of the global environment (Krupic, Bauza, Burton, Barry, & O'Keefe, 2015). However, up to the point when the work described in this thesis had started, the field lacked a systematic probing of the hexagonal stability of single firing fields.

Cells beyond space

As mentioned before, humans and animals are not necessarily restricted to navigation of physical space. Recent physiological findings are certainly consistent with this idea. Neurons encoding time have been found in the hippocampal formation (MacDonald, Lepage, Eden, & Eichenbaum, 2011; Tsao et al., 2018)
In fact the same neurons known to encode physical space may encode "space" in other domains. Studies done in monkeys has shown that grid cells can encode visual space in a similar way as physical space (Killian, Jutras, & Buffalo, 2012). While both Place cells and Grid Cells have been show to encode similarly physical space and auditory frequency space (Aronov, Nevers, & Tank, 2017).

The hippocampus has been also found to host cells representing concepts, like that of the actress Jenifer Aniston (Quiroga, Reddy, Kreiman, Koch, & Fried, 2005).
All of this implies that the same structures and even cells used to navigate space could be used to navigate other domains.

Navigating a game: Prefrontal Cortex

Prefrontal cortex is an area of the neocortex which has been of great interest in the study of human cognitive capabilities. It has been ascribe a role in human cognitive benchmarks like language (Gabrieli, Poldrack, & Desmond, 1998), imagination (Schacter, Addis, & Buckner, 2007), emotional processing

(Etkin, Egner, & Kalisch, 2011), complex decision making (Miller & Cohen, 2001). As an area, encompassing multiple subareas, the prefrontal cortex has been selected in evolution and has expanded exceptionally in great apes (Smaers, Gómez-Robles, Parks, & Sherwood, 2017).
Although initially controversial, both anatomical and functional findings show that rats have a prefrontal cortex with certain functional homologies to medial and orbital areas in primates, as well as some of the features characteristic of primate dorsolateral prefrontal cortex (Uylings, Groenewegen, & Kolb, 2003).

In rats, prefrontal cortex has been linked to decision making and evidence accumulation (Feierstein, Quirk, Uchida, Sosulski, & Mainen, 2006; Kepecs, Uchida, Zariwala, & Mainen, 2008). In a similar fashion as has been described for non-human primates (Romo, Brody, Hernández, & Lemus, 1999), where medial prefrontal cortex (mPFC) neurons encode both the quality of the stimulus, and the decision taken by the animal. Even though mPFC is involved in task encoding (Feierstein et al., 2006; Kepecs et al., 2008; Romo et al., 1999), individual cells are said to be encoding more than one feature of the task, that include the intensity or the decision. This mixed selectivity (Raposo, Kaufman, & Churchland, 2014) has been addressed by dimensionality reduction methods like LDA, PCA, and demixed PCA (Kobak et al., 2016). These methods are able demix the different task dependencies encoded by the population.

Further on, it was found that in rats learning two tasks with different rules, there is a reset of network activity in mPFC in the transition from one task two another (Karlsson, Tervo, & Karpova, 2012). This implies an important role for mPFC in switching between behavioral strategies.

Recent work has explored the important relation between decision making and spatial navigation. Medial prefrontal cortex has been found to prospectively encode the navigational behavior, as their firing in a path alternation task is trajectory dependent. Medial prefrontal cortex may be contributing with planning future goal directed trajectories by controlling hippocampal activity (Ito, Zhang, Witter, Moser, & Moser, 2015).

Even though in humans, medial prefrontal cortex has an important role in social cognition, and even in the development of such cognition early in life (Grossmann, 2013) (Grossmann, 2013). Little is known about the role of mPFC

in the social life of rats. However two recent papers describe mPFC cell responses to the approach of a conspecific (Lee et al., 2016; Murugan et al., 2017).

These recent findings, together with the remarkable discovery of social place cells in bats (Omer, Maimon, Las, & Ulanovsky, 2018) and rats (Danjo, Toyoizumi, & Fujisawa, 2018) in the hippocampus, suggests that the interaction between medial prefrontal cortex and hippocampus could be about more than just space, but allow the animals to also navigate in relation to the behavior of others. This includes navigating space itself but also points to the possibility these structures contribute other "spaces", the personal space of others, or even an abstract space defined by social relationships (Schafer & Schiller, 2018) and even circumstantial social roles.
In this thesis, we attempt a first look at medial prefrontal cortex in the context of'Hide and Seek' a sophisticated role-playing game that involves knowing the position of on self, the configuration of an environment and the position of another individual.

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Outline of this thesis

The original work of this thesis is organized in 3 chapters with increasing behavioral complexity. Chapter I, addresses the structure and function of the parasubiculum using a variety of techniques and a standard open field behavioral paradigm. Chapter II, explores the encoding of the animals home in parahippocampal regions. Chapter III, establishes for the first time the use of Hide and Seek in rats as interspecies game with a spatial navigation component and looks into neural correlates of the game in the medial prefrontal cortex.

Each chapter is embedded between a short overview working as a sort of foreword, and a perspective projecting into the future of the field. The thesis is wound up by a broad general discussion and perspectives for the future.

CHAPTER I

Structure Function Relations of the Parasubiculum

Overview

In the past decade the field of spatial neuroscience has achieved a remarkable level of understanding about the functional classes of cells that constitute the navigational substrate of brains. The discovery of place cells, head direction cells, border cells and grid cells has given us a fair amount of insight. However, little is known about how these functional cells are embedded in the anatomy and circuitry of the parahippocampal areas. In this chapter we looked at the parasubiculum with a variety of techniques in order to get deeper understanding of its role in the circuitry controlling spatial navigation.

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Functional architecture of the rat parasubiculum

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Abstract

The parasubiculum is a major input structure of layer 2 of medial entorhinal cortex, where most grid cells are found. Here we investigated parasubicular circuits of the rat by anatomical analysis combined with juxtacellular recording/labeling and tetrode recordings during spatial exploration. In tangential sections, the parasubiculum appears as a linear structure flanking the medial entorhinal cortex medio-dorsally. With a length of ~ 5.2 mm and a width of only ~ 0.3 mm (approximately one dendritic tree diameter) the parasubiculum is both one of the longest and narrowest cortical structures. Parasubicular neurons span the height of cortical layers 2 and 3 and we observed no obvious association of deep layers to this structure. The "superficial parasubiculum" (layers 2 and 1) divides into ~ 15 patches, whereas deeper parasubicular sections (layer 3) form a continuous band of neurons. Anterograde tracing experiments show that parasubicular neurons extend long 'circumcurrent' axons establishing a 'global' internal connectivity. The parasubiculum is a prime target of GABAergic and cholinergic medial septal inputs. Other input structures include the subiculum, the presubiculum and anterior thalamus. Functional analysis of identified and unidentified parasubicular neurons shows strong theta-rhythmicity of spiking, a large fraction of head-direction selectivity (50%, 34/68) and spatial responses (grid, border and irregular spatial cells, 57%, 39/68). Parasubicular output preferentially targets patches of calbindin-positive pyramidal neurons in layer 2 of medial entorhinal cortex, which might be relevant for grid cell function. These findings suggest the parasubiculum might shape entorhinal theta-rhythmicity and the (dorsoventral) integration of information across grid scales.

Introduction

The analysis of spatial discharge patterns in hippocampal and parahippocampal brain regions is a remarkable success story (Moser et al., 2008; Moser and Moser, 2013, Burgess, 2014). Extracellular recordings revealed an astonishing degree of complexity, abstractness, but also identified clear behavioral correlates of discharge patterns, such as place, head-direction, border and grid cells. Along with the exploration of discharge properties, anatomists delineated in great detail the basic circuitry of the hippocampal formation (Amaral and Witter, 1989; van Strien et al., 2009).

The detailed data available about certain parts of the hippocampal formation – such as dorsal CA1 in the rodent – should not blind us for gaps in our knowledge about less 'classic' hippocampal processing nodes. The parasubiculum is one such structure that lies beyond the classic tri-synaptic hippocampal loop (Andersen et al., 1971) and has been investigated relatively little. This parahippocampal region provides massive input to layer 2 of medial entorhinal cortex (van Groen and Wyss, 1990; Caballero-Bleda and Witter, 1993, 1994) and shows prominent expression of markers for cholinergic activity (Slomianka and Geneser, 1991). Early physiological analysis described a small fraction of place-responsive cells in the parasubiculum (Taube, 1995) and subsequent extracellular recordings have also identified head-direction, border and grid responses among parasubicular neurons (Cacucci et al., 2004; Boccara et al., 2010).

Both from a physiological and an anatomical perspective, the parasubiculum is somewhat difficult to study. First, the small size of the parasubiculum complicates recordings and tracer injections. Second, the parasubicular position (on the caudal edge of the parahippocampal lobe wrapping around entorhinal cortex, which goes along with a strong bending of the cortical sheet) greatly complicates the delineation of the parasubiculum. Here we aimed for a comprehensive description of parasubicular circuits by a combined anatomical and functional approach (Burgalossi et al., 2011; Tang et al., 2014a). Specifically, we were interested in how parasubicular circuits relate to pyramidal and stellate neuron microcircuits in layer 2 of medial entorhinal cortex (MEC, Ray et al., 2014; Tang et al., 2014b).

In our current analysis we investigate four issues: First, we delineate the location, shape, laminar organization and internal structure of parasubiculum.

Second, we investigate the sources of parasubicular inputs, as well as the targets of parasubicular outputs. Third, we assess spatial discharge patterns of parasubicular neurons by juxtacellular recording/labeling and tetrode recordings in freely moving rats. Fourth, we assess the temporal discharge patterns of identified and unidentified parasubicular neurons, and how this might relate to anatomical connectivity.

Results

Geometry of the parasubiculum

In our initial analysis we sought to determine the general organization of the parasubiculum (PaS). Tangential sections (parallel to the pial surface of the MEC, see Methods) of the cortical sheet stained for acetylcholine esterase activity (Figure 1A, left) or calbindin immunoreactivity (Figure 1A, right) provide a particularly clear overview of the spatial extent of the parasubiculum. Consistent with findings from previous stud–ies (Geneser, 1986; Slomianka and Geneser, 1991), we find that the parasubiculum shows prominent acetylcholine esterase activity (Figure 1A, left). The parasubiculum can also be identified by an absence of calbindin immunoreactivity (Figure 1A, left; Fujise et al., 1995; Boccara et al., 2010). Further subdivisions of the parasubiculum have been suggested (Blackstad, 1956). Our data refer to the calbindin free area surrounding the MEC outlined in Figure 1A left and highlighted in light-blue in Figure 1B (possibly related to 'parasubiculum b' in the terminology of Blackstad, 1956). Laterally contiguous to the parasubiculum one observes a thin strip of cortex containing numerous calbindin-positive neurons (Figure 1A right, Figure 1B red 'calbindin stripe').
As shown in Figure 1A-B, and quantified in Figure 1C, the parasubiculum forms a fairly narrow (310 ± 83 μm width, N = 10), but very elongated (5.190 ± 0.485 mm length, N = 10) continuous curved stripe, which flanks the medial entorhinal cortex from its medial to dorso-lateral side. The lateral part of the parasubiculum, dorsal to the medial entorhinal cortex, is narrower than the medial part. This may explain why this part of the parasubiculum has not been classified as such in most previous studies (Boccara et al., 2010; Ding, 2013). Other histological markers, such as cytochrome-oxidase activity, or soma morphologies, as visualized from Nissl stains (Burgalossi et al., 2011) also delineated the parasubiculum in the same way as shown in Figure 1 (not shown). Similarly, parasagittal sectioning angles delineate the same outlines

of the parasubiculum. We conclude that the parasubiculum has a linear structure with a narrow width.

We also investigated the laminar structure of the parasubiculum. Consistent with our previous conclusions (Burgalossi et al., 2011), we did not find direct evidence for a clear association of deep layers with the parasubiculum. For example, following tracer injections in the superficial parasubicular layers, we did not observe back-labeled neurons in the adjacent deep layers, even when we observed back-labeled neurons as distant as the subiculum (data not shown). Hence, we speculate that deep layers close to the parasubiculum might not be part of this structure but could rather be associated to the neighboring medial entorhinal cortex or the presubiculum (Mulders et al., 1997).

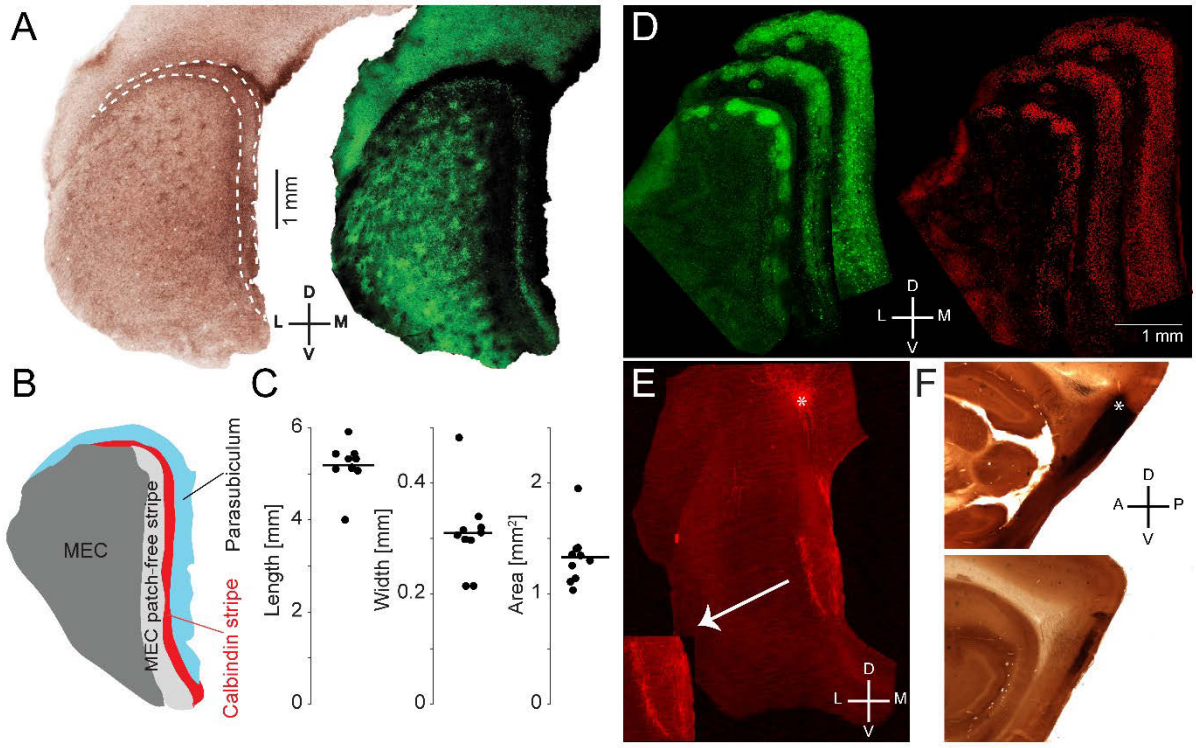


Figure 1. Shape and internal structure of the parasubiculum.

(A) Left: Tangential section stained for acetylcholinesterase activity (dark precipitate). The shape of the parasubiculum is outlined (white dashed line) coinciding with high acetylcholinesterase activity. Right: Tangential section (same section as in Figure 1A left) processed for calbindin immunoreactivity (green), the shape of the parasubiculum is negatively outlined by an absence of calbindin immunoreactivity.

(B) Schematic of the parasubiculum (light blue) and adjacent medial entorhinal cortex (MEC) subdivisions.

(C) Quantification of parasubiculum size in 10 hemispheres: Length, width and area.

(D) Tangential sections stained for parvalbumin (PV) immunoreactivity (green, left) and NeuN immunoreactivity (red, right). The parasubiculum stands out by its intense staining. Three sections are shown, the left one is most superficial (closest to the pia), middle and right are progressively deeper. Note how the patchy structure of the superficial parasubiculum is replaced by continuous cell band in deeper sections.

(E)Tangential sections of the parasubiculum showing the injection site of BDA tracer (red fluorescence) and anterogradely-traced circumcurrent axons (according to the terminology of Burgalossi et al., 2011) extending throughout the parasubiculum (see also magnified inset on the left). Injection site is marked with a white asterisk.

(F) Parasagittal sections of the parasubiculum (top), parasubiculum and MEC (bottom) after the injection of larger amounts of BDA (tracer, dark color). The tracer completely fills the parasubiculum and stains layer 2 of the MEC. Injection site is marked with a white asterisk.

(A) and (B) were modified from (Ray et al., 2014).

D = dorsal, L = lateral, M = medial, V = ventral

Internal structure of the parasubiculum

Consistent with our previous observations (Burgalossi et al., 2011), we found the superficial parts of the parasubiculum (corresponding to layers 1 and 2) can be divided into ~15 large patches with a diameter around 500 µm each. These patches can be revealed in superficial tangential sections (Figure 1D, left) by parvalbumin (PV)-immunoreactivity and by cell density visualized by

NeuN immunoreactivity (Figure 1D, right). However, the deeper parts of the parasubiculum (corresponding to layer 3) were not obviously divided into patches (Figure 1D).

Injections of the anterograde tracer Biotinylated Dextrane Amine (BDA, 3000 MW) showed that parasubicular neurons extend long axons throughout the full length of the parasubiculum (Figure 1E), consistent with previous evidence from single-cell microcircuits (Burgalossi et al., 2011). In the latter work, these axons were termed "circumcurrent", as they appeared to inter-connect parasubicular patches. As a consequence of this internal connectivity, a single tracer injection could label the full extent of the parasubiculum (Figure 1F). This is a remarkable feature of the parasubiculum not seen in the medial entorhinal cortex. Thus, analysis of the internal structure of parasubiculum indicates both modularity and global connectivity.

Inputs to the parasubiculum

Of particular interest for hippocampal function are the inputs from the medial septum, which are of critical importance to grid cell activity (Brandon et al., 2011; Koenig et al., 2011). We first sought to determine the patterns of GABAergic inputs from the medial septum, which are thought to play a critical role in theta-rhythm generation (Mitchell et al., 1982; Buzsáki, 2002; Hangya et al., 2009; Brandon et al., 2011; Koenig et al., 2011). To this end, we performed viral injections in the medial septum in PV-Cre mice (see Methods), and expressed GFP selectively in GABAergic septal neurons (Figure 2A,B). As shown in Figure 2A, the parasubiculum is an area within the hippocampal formation, which receives a comparatively dense innervation from GABAergic medial septal neurons (as quantified by normalized fluorescence levels, Mean values (n=3), PaS = 1.5 ± 0.23, MEC = 1.0 ± 0.05, CA1-3 = 1.2 ± 0.06, PreS = 1.0 ± 0.04. Figure 2C). As we already noted earlier, there is also a prominent expression of cholinergic activity markers (Figure 1A and Figure 2D) in line with in vitro work showing robust response of parasubicular neurons to muscarinic activation (Glasgow and Chapman, 2013). Taken together, these data point towards a strong medial septal drive to parasubicular neurons, likely contributing to strong theta rhythmicity in the parasubiculum (Burgalossi et al., 2011; see below).

By retrograde-tracer injections, we also identified parasubiculum-projecting neurons in the anterior thalamus, subiculum and presubiculum. The findings

are consistent with the earlier conclusions of previous authors (Köhler, 1985; van Groen and Wyss, 1992; Honda and Ishizuka, 2004) and are therefore not shown.

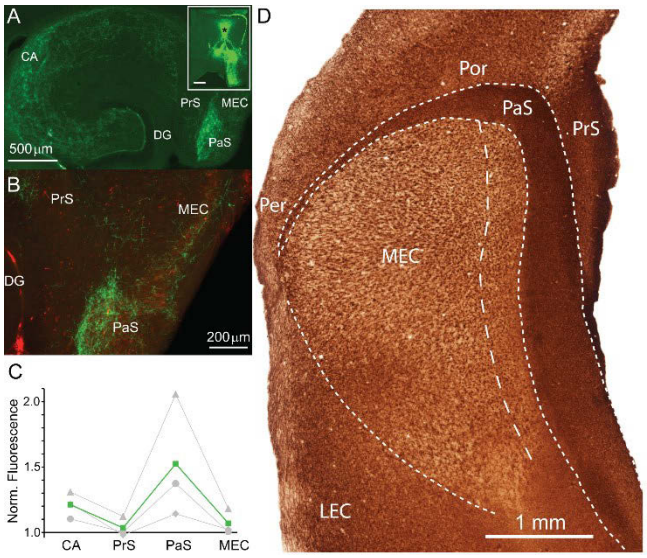


Figure 2. Parasubiculum receives GABAergic and cholinergic inputs.

(A) Horizontal sections showing that the parasubiculum contains the densest projection in the hippocampal formation of GFP-positive, putative parvalbuminergic fibers. Deriving from injection of AAV into the medial septum (inset, asterisk) of mice expressing Cre recombinase under the parvalbumin promoter. This dense projection pattern was seen in 3/3 injected mice. Note in this brain also olfactory and accessory olfactory areas were labelled unilaterally.

(B) PV immunostaining (red) marks the extent of the parasubiculum (as shown in Figure 1D Left). Note the higher density of GABAergic medial septal fibers (green) within the parasubiculum.

(C) Normalized fluorescence intensity levels relative to dentate gyrus (n = 3 mice, different grey symbols represent the different mice). Mean normalized fluorescence shown as green squares.

(D) Tangential section showing high levels of Acetylcholinesterase (AChE) in the parasubiculum.

MEC= medial entorhinal cortex, PrS= presubiculum, DG= dentate gyrus, LEC= lateral entorhinal cortex, PaS= parasubiculum, Por= postrhinal cortex, Per= perirhinal cortex, CA= cornus ammonis.

Outputs from the parasubiculum

Previous work showed that the parasubicular axons innervate layer 2 of the medial entorhinal cortex (van Groen and Wyss, 1990; Caballero-Bleda and Witter, 1993, 1994; but see Canto et al., 2012). Recent work showed that principal neurons in layer 2 of medial entorhinal cortex segregate into stellate and pyramidal cell subnetworks, which can be differentiated by the calbindin immunoreactivity of the pyramidal neurons (Varga et al., 2010). Layer 2 pyramidal neurons are arranged in a hexagonal grid, show strong theta-rhythmic discharges (Ray et al., 2014) and might preferentially contribute to the grid cell population (Tang et al. 2014b; but see Sun et al., 2015). To determine if parasubicular inputs target a specific subpopulation of neurons in layer 2 of medial entorhinal cortex, we performed fine scale injections of anterograde tracers in the dorsal parasubiculum, combined with visualization of calbindin patterns (Figure 3). As shown in Figure 3, tangential sections through layer 2 with calbindin immuno-staining revealed a regular organization of patches of pyramidal neurons (Ray et al., 2014). Surprisingly, these patches were selectively innervated by parasubicular afferents (Figure

3A-B), which targeted the center of patches (Figure 3C). This indicates that parasubicular axons may preferentially target layer 2 pyramidal neurons of medial entorhinal cortex, which may in turn contribute to the strong theta rhythmicity in these neurons (Ray et al., 2014.)

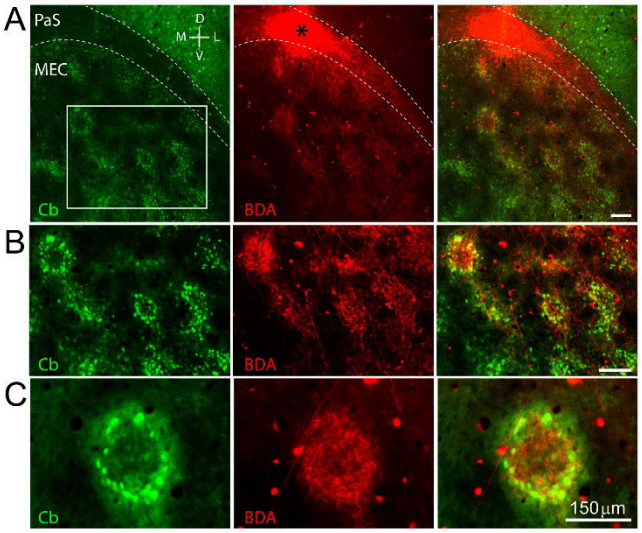


Figure 3. Parasubicular axons target layer 2 pyramidal cell patches in medial entorhinal cortex.

(A) Left, tangential section stained for calbindin (green) revealing patches of calbindin-positive pyramidal neurons. Middle, same section as left processed to reveal the tracer BDA (red). The location of the parasubicular injection site is marked with a black star. Right, overlay. Scale bar, 150 microns

(B) Same as (A) but higher magnification. Scale bar, 150 microns

(C) High magnification view of a single patch. D = dorsal, L = lateral, M = medial, V = ventral.

Identification of functional cell types in the parasubiculum

In comparison to its major target structure - the entorhinal cortex - limited information is currently available about the spatial discharge properties in the parasubiculum (Taube, 1995; Cacucci et al., 2004; Boccara et al., 2010). To address this issue, we juxtacellularly recorded and labeled neurons (n = 16) in the parasubiculum of freely moving rats trained to explore 2D environments (Tang et al., 2014a) A representative recording from an identified parasubicular neuron is shown in Figure 4A. This neuron had divergent sideward-directed dendrites (seen from the top), was situated in the dorsal part of the parasubiculum (Figure 4A, right) and discharged in spike bursts strongly entrained by the theta rhythm (Figure 4B). Theta rhythmicity of spiking was revealed by the spiking autocorrelogram (Figure 4C, left) and the spikes were also strongly locked to local theta oscillations (Figure 4C, right). The neuron discharged along the border of the enclosure (Figure 4D, left), a defining feature of border activity (Solstad et al., 2008) and showed head-direction selectivity (Figure 4D, right).

In line with previous observations in linear mazes (Burgalossi et al., 2011), many juxtacellularly-recorded neurons showed head-direction selectivity. A representative neuron is shown in Figure 4E. This neuron was situated in the medial part of the parasubiculum (Figure 4E, right) and also discharged in

bursts with strong theta rhythmicity (Figure 4F, right). The spiking autocorrelogram also revealed a strong theta rhythmicity (Figure 4G, left), and the spikes were strongly locked to local theta oscillations (Figure 4G, right). Spikes were fired throughout the enclosure without obvious spatial modulation (Figure 4H, left), but showed a clear head-direction preference (Figure 4H, right).

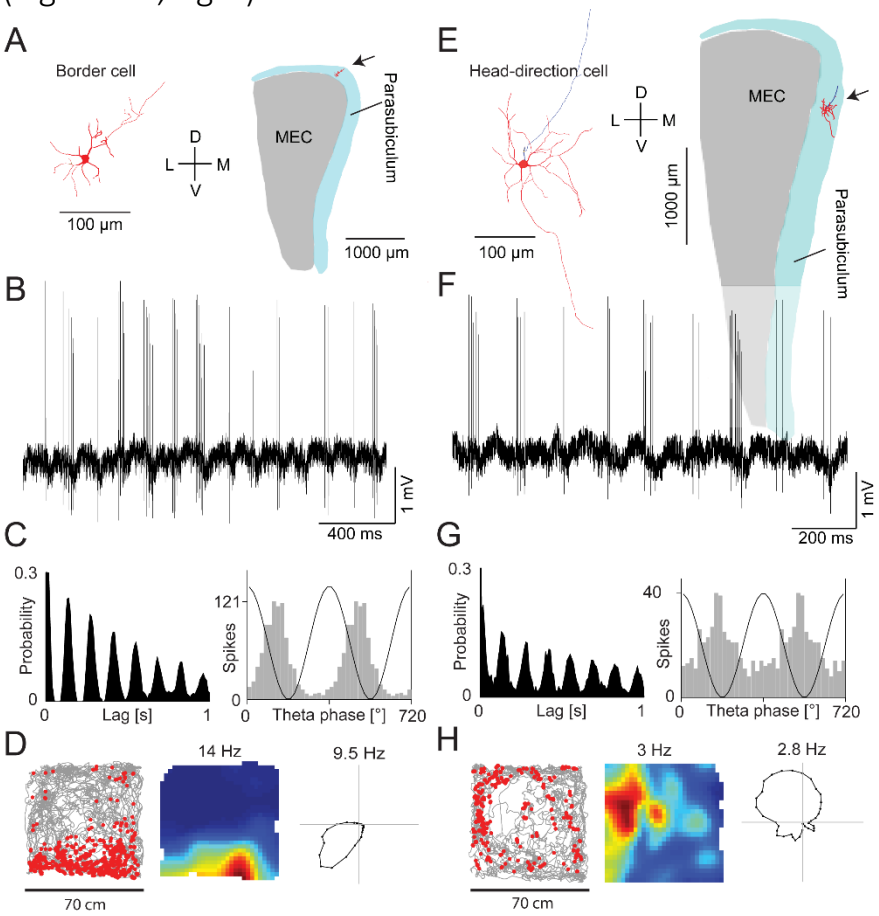


Figure 4. Physiology of identified parasubicular neurons.

(A) Left: Reconstruction of a border cell juxtacellularly-recorded and identified in a rat exploring a 2D environment (70 x 70 cm). Reconstructed dendrites and axon shown in red and blue, respectively. Scale bar, 100 µm. Right: Schematic of the location the cell in the parasubiculum (arrow). The cell is located in the dorsal band of parasubiculum (blue), close to medial entorhinal cortex (grey). Scale bar, 1000 µm.

(B) Representative raw traces of the recorded cell shown in (A). Note the prominent theta rhythm in LFP and theta-modulated firing of the recorded cell.

(C) Left: Autocorrelogram of spike discharges for the cell shown in (A). Right: Theta-phase histogram of spikes for the cell shown in (A). For convenience, two repeated cycles are shown. The black sinusoid is a schematic local field potential theta wave for reference.

(D) Spike-trajectory plot (left), rate map (middle) revealing the border firing. Spike-trajectory plot: red dots indicate spike locations; grey lines indicate the rat trajectory. Rate map: red indicates maximal firing rate, value noted above. For this cell, the border score is 0.86. Right: Polar plot of the cell's head-direction tuning. Value indicates maximum firing rate to the preferred direction.

(E-H) Same as (A-D) for an identified head-direction cell.

D = dorsal, L = lateral, M = medial, V = ventral.

Spatial firing properties of parasubicular neurons

By combining juxtacellularly-recorded and identified parasubicular neurons with verified recording sites of single-cell and tetrode recordings (see Methods), we could provide a more comprehensive characterization of functional cell types in parasubiculum. In line with previous work (Boccara et al., 2010), we observed border discharges (9%, 6/68, Figure 4D & 5A), grid discharges (9%, 6/68, Figure 5B), strong head-direction selectivity (50%, 34/68, Figure 4H & 5C) and a substantial proportion of irregular spatial discharges (40%, 27/68) (cells not shown). This last group contains cells with significant spatial information content (Skaggs et al., 1993; see Methods) but that do not meet grid or border inclusion criteria (see Methods).

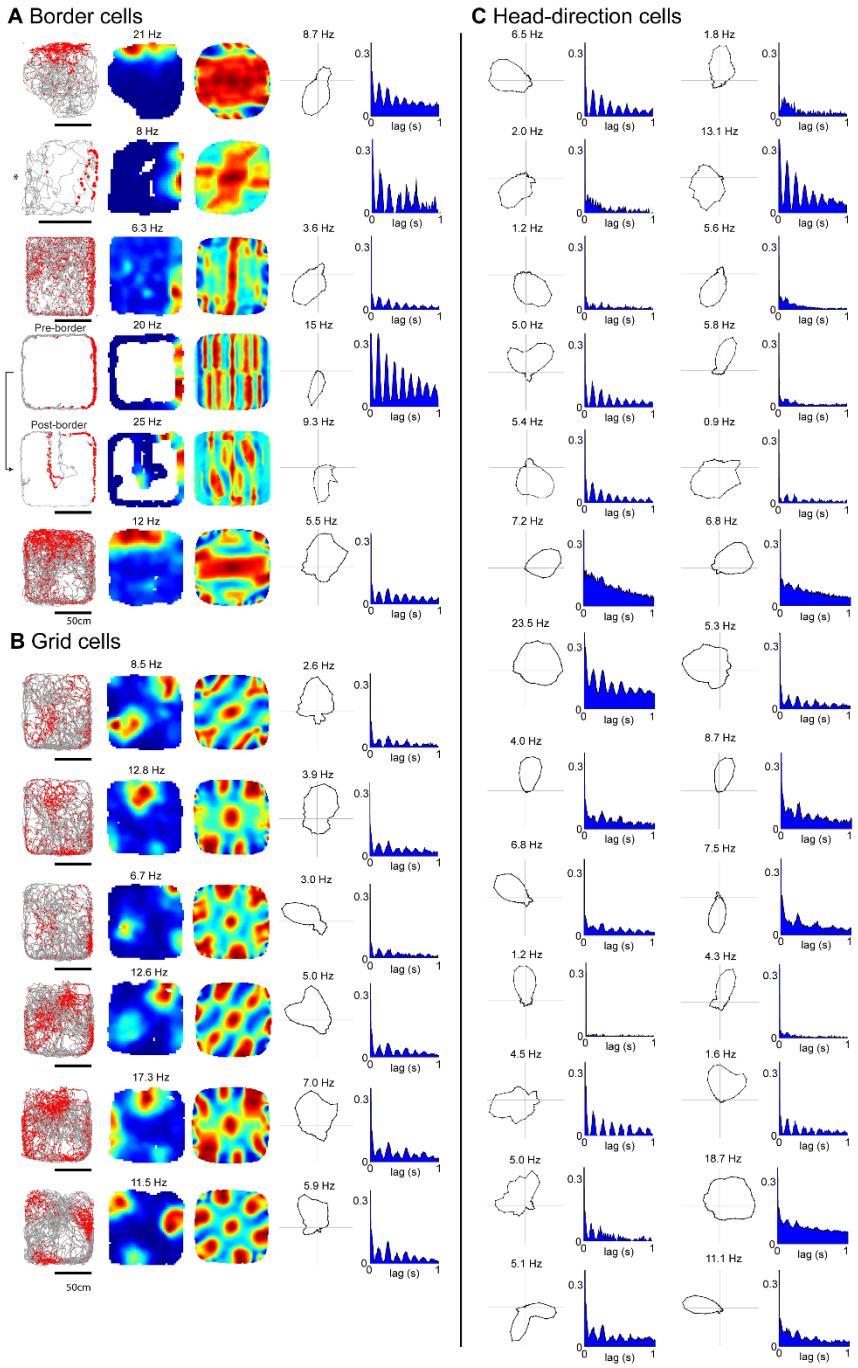

A Border cells
21 Hz
8.7 Hz
8 Hz
6.3 Hz
3.6 Hz
Pre-border
20 Hz
15 Hz
Post-border
25 Hz
9.3 Hz
12 Hz
5.5 Hz
50cm
B Grid cells
8.5 Hz
2.6 Hz
12.8 Hz
3.9 Hz
6.7 Hz
3.0 Hz
12.6 Hz
5.0 Hz
17.3 Hz
7.0 Hz
11.5 Hz
5.9 Hz
50cm
C Head-direction cells
6.5 Hz
1.8 Hz
2.0 Hz
13.1 Hz
1.2 Hz
5.6 Hz
5.0 Hz
5.8 Hz
5.4 Hz
0.9 Hz
7.2 Hz
6.8 Hz
23.5 Hz
5.3 Hz
4.0 Hz
8.7 Hz
6.8 Hz
7.5 Hz
1.2 Hz
4.3 Hz
4.5 Hz
1.6 Hz
5.0 Hz
18.7 Hz
5.1 Hz
11.1 Hz
lag (s)

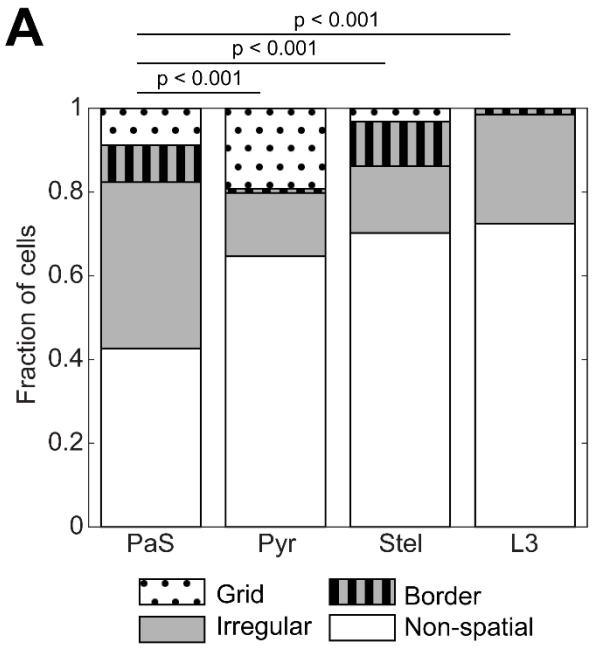
Figure 5. Border, grid and head-direction firing properties of parasubicular neurons.

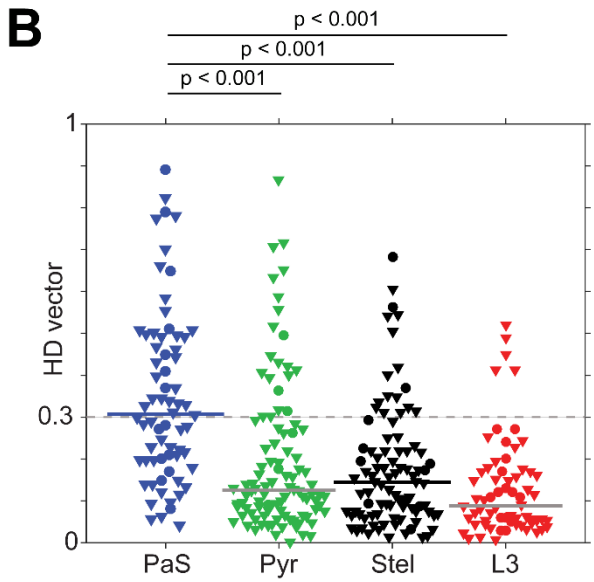
(A) Parasubicular neurons classified as border cells. From left to right: Spike-trajectory plot, rate map, two-dimensional spatial autocorrelation, angular tuning (shown only for head-direction selective cells) and spike autocorrelogram. Numbers above the rate map indicate maximum firing rate. Numbers above the angular tuning map indicate maximum firing rate at the preferred direction. Scale bar below the Spike-trajectory plot corresponds to 50cm. An asterisk marks a border cell recorded in a 70x70 cm arena. Pre-border and Post-border refer to the border test (recording of the same cell before and after the introduction of an additional wall into the arena).

(B) Parasubicular neurons classified as grid cells (same panels as in A)

(C) Parasubicular neurons classified as Head Direction cells (see Methods). Left: angular tuning. Right: spike autocorrelogram. Conventions as in (A).

Next, we compared the spatial discharge properties of the parasubiculum to those of identified and putative MEC Layer 2 pyramidal and stellate neurons (Tang et al., 2014b) as well as neurons recorded in MEC Layer 3 (Tang et al. 2015). We found significantly more spatial responses in the parasubicular neurons than in the other cell types (Figure 6A, all $p < 0.01$, χ^2-test with Bonferroni-Holm correction: 39/68 PaS, $\chi^2 = 7.91$ vs. 35/99 Pyr, $\chi^2 = 12.4$ vs. 28/94 Stel, $\chi^2 = 11.1$ vs. 19/66 L3). We also observed a strong head directionality of parasubicular neurons, in line with previous observations from linear track recordings (Burgalossi et al., 2011). At the population level, the median head-direction vector of all parasubicular neurons was 0.31, much larger than in MEC Layer 2 (0.12 in Pyramidals; 0.14 in Stellates) and Layer 3 (0.09 in Layer 3 cells; Figure 6B, all $p < 0.001$, Mann-Whitney U-tests with Bonferroni-Holm correction: z(Pyr) = 5.54, z(Stel) = 5.79, z(L3) = 7.01). Similarly, the proportion of neurons classified as head-direction cells was also considerably larger than in MEC Layer 2 and Layer 3 (Figure 6C, all $p < 0.001$, χ^2-test with Bonferroni-Holm correction: χ^2(Pyr)= 17.7, χ^2(Stel) = 28.8, χ^2(L3) = 30.7)


A
p < 0.001
p < 0.001
p < 0.001
Fraction of cells
1
0.8
0.6
0.4
0.2
0
PaS
Pyr
Stel
L3
Grid
Border
Irregular
Non-spatial


B
p < 0.001
p < 0.001
p < 0.001
HD vector
1
0.3
0
PaS
Pyr
Stel
L3

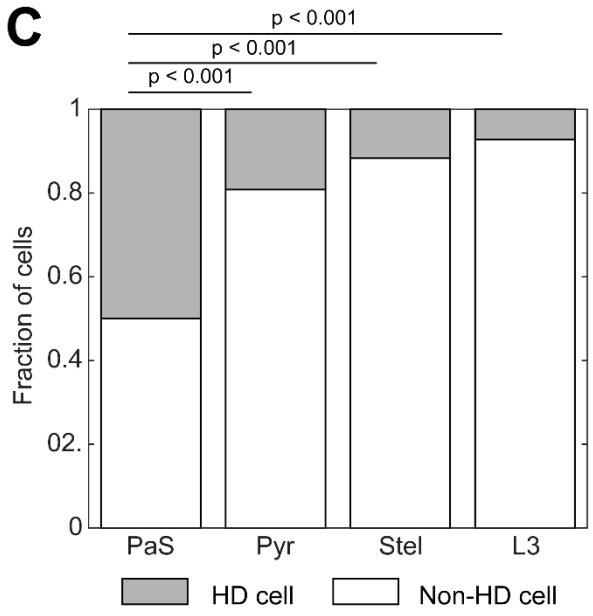

C
p < 0.001
p < 0.001
p < 0.001
Fraction of cells
1
0.8
0.6
0.4
02.
0
PaS
Pyr
Stel
L3
HD cell
Non-HD cell

Figure 6. Border and head-direction firing properties of parasubicular neurons.

Data from layer 2 and layer 3 of medial entorhinal cortex (MEC) come from the work of (Tang et al., 2014b) and (Tang et al., 2015) and are shown for comparison.

(A) Comparison of fractions of spatial discharges for parasubiculum, MEC L2 pyramidal, MEC L2 stellate and MEC L3 neurons. Parasubicular neurons show large fraction of significantly spatially modulated cells: grid cells, border cells and spatially irregular cells. (χ^2 test with Bonferroni-Holm correction).

(B) Comparison of head-direction (HD) vector lengths for parasubiculum (blue), MEC L2 pyramidal (green), MEC L2 stellate (black) and MEC L3 (red) neurons. Parasubicular neurons show significantly higher average HD vector length than all others (Mann-Whitney U-tests with Bonferroni-Holm correction). Lines indicate medians. Horizontal dotted line at 0.3 marks the threshold for HD classification.

(C) Comparison of fractions of head-direction cells (HD cells) for parasubiculum, MEC L2 pyramidal, MEC L2 stellate and MEC L3 neurons. Parasubicular neurons show significantly higher percentage of HD cells (χ^2 test with Bonferroni-Holm correction).

Theta modulation of parasubicular neurons

As shown in representative neurons (Figures 4 and 5), the large majority of parasubicular neurons showed strong theta-rythmicity, as revealed by autocorrelation of spike trains (Figure 7A, see also Tang et al., 2014b and Tang et al., 2015). Parasubicular neurons were also strongly locked to local field potential theta oscillations, which is known to be in phase with MEC theta (Glasgow and Chapman, 2007; Figure 7B). On average, theta rhythmicity was stronger in parasubicular neurons than in identified layer 2 stellates and layer 3 neurons (Both $p < 0.01$, Mann-Whitney U-test, z(Stel) = 3.19, z(L3) = 8.39, Figure 7C; MEC cells from Tang et al., 2014b and Tang et al., 2015). Identified parasubicular neurons tended to have a higher theta rhythmicity than identified layer 2 neurons (juxtacellularly recorded cells, $p = 0.0116$, Mann-Whitney U-test), but this difference did not reach statistical significance when tetrode-units were included in the sample of parasubicular neurons. Theta-phase locking strength (mean (circular) vector length, see Methods) of parasubicular neurons was similar to that of MEC layer 2 pyramidal neurons ($p > 0.05$, Mann-Whitney U-test: z = -0.89, Figure 7D), and significantly stronger than that of layer 2 stellates and layer 3 neurons (Both $p<0.001$, Mann-Whitney U-test: z(Stel) = 3.73, z(L3) = 7.83, Figure 7D; MEC cells from Tang et al., 2014b and Tang et al., 2015).

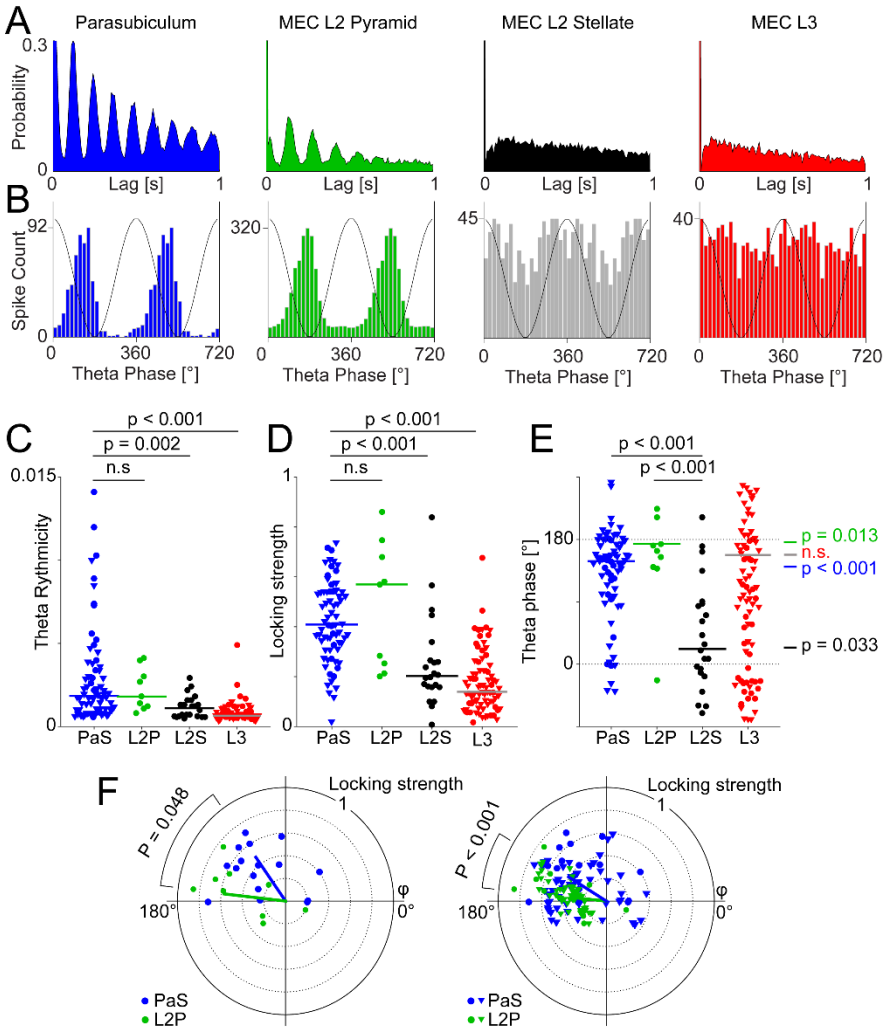

A
Parasubiculum
MEC L2 Pyramid
MEC L2 Stellate
MEC L3
Probability
0.3
0
Lag [s]
B
Spike Count
92
320
45
40
Theta Phase [°]
C
p < 0.001
p = 0.002
n.s
Theta Rythmicity
0.015
PaS L2P L2S L3
D
p < 0.001
p < 0.001
n.s
Locking strength
1
E
p < 0.001
p < 0.001
Theta phase [°]
180
p = 0.013
n.s.
p < 0.001
p = 0.033
F
Locking strength
P = 0.048
P < 0.001
180°
0°
φ
PaS
L2P

Figure 7. Theta modulation of parasubicular neurons compared to superficial medial entorhinal cortex.

Data from layer 2 and layer 3 of medial entorhinal cortex (MEC) come from the work of (Tang et al., 2014b) and (Tang et al., 2015) and are shown here for comparison.

Representative autocorrelograms of spike discharges of identified neurons recorded from parasubiculum (blue), MEC L2 pyramidal (L2P, green), MEC L2 stellate (L2S, black) and MEC L3 (red) neurons.

Theta-phase histogram of spikes for the neurons shown in (A). For convenience, two theta-cycles are shown. The black sinusoid is a schematic local field potential theta wave for reference.

Comparison of the power of theta rhythmicity in parasubiculum (blue), MEC L2 pyramid (L2P, green), MEC L2 stellate (L2S, black) and MEC L3 (L3, red) neurons. Parasubiculum neurons show significantly stronger theta rhythmicity than MEC L2 stellate and MEC L3 neurons (Kruskal-Wallis test with Bonferroni correction). Lines indicate medians.

Comparison of the theta-phase locking strength (abbreviated in the Figure as Locking Strength) for the neurons shown in in (C). Parasubicular and MEC L2 pyramidal neurons show significantly higher theta-phase locking than MEC L2 stellate and MEC L3 neurons (Kruskal-Wallis test with Bonferroni-Holm correction; significant differences between L2P and L2S has been shown in Ray et al., 2014). Lines indicate medians.

Comparison of the preferred theta phase for the neurons shown in (C). Parasubicular and MEC L2 neurons show significant preferred theta phases, while MEC L3 neurons do not (Rayleigh test for non-uniformity with Bonferroni-Holm correction: colored p-values on the right side; Watson-Williams test for equal means with Bonferroni-Holm correction: black lined p-values top). Colored lines indicate circular means.

Polar plots of preferred theta phase (theta peak = 0°) and theta-phase locking strength ((Locking Strength; Rayleigh vector, 0-1) for parasubiculum (blue), MEC L2 pyramidal (green). Left: only identified neurons. Right: Identified neurons and tetrode recordings. dots = identified neurons, triangles = tetrode units, line = (mean direction, median strength of locking).

Notably, at the population level parasubicular and MEC layer 2 pyramidal and stellate neurons showed distinct preferred theta phases (all $p < 0.05$, Rayleigh test for non-uniformity: z(PaS) = 29.5, z(Pyr) = 4.07, z(Stel) = 3.36, Figure 7E; MEC cells from Tang et al., 2014b and Tang et al., 2015). When we compared the preferred phase of identified MEC Layer 2 pyramids and identified parasubicular neurons, we found that the parasubicular neurons preferred an earlier theta phase (slightly before the trough, Figure 7F left, $p = 0.048$, Watson-Williams test for equal circular means: $F = 4.34$). When we included all non-identified juxta and tetrode recordings of parasubicular and putative MEC Layer 2 pyramidal neurons, this difference remained statistically significant (Figure 7F right, 155° vs. 174° $p = 0.0000085$, Watson-Williams test for equal circular means: $F = 22.7$). Since tetrode recordings of MEC Layer 2 were assigned their putative cell identity based on their temporal spiking properties, we wondered whether this might have biased the comparison of preferred theta phase. However, two indications suggest that this was not the case: *(i)* in the identified dataset, we had not excluded any MEC Layer 2 neurons which locked before the trough (see Tang et al. 2014b). *(ii)* Even when we applied the same classifier to all parasubicular neurons and only compared MEC Layer 2 putative pyramids to parasubicular neurons, which would have been classified as putative pyramids, the difference in preferred phase was

still trending towards significance ($p = 0.076$, Watson-Williams test for equal circular means: $F = 3.21$).

The strong theta-phase locking strength and theta rhythmicity of both parasubicular neurons and layer 2 pyramidal (but not stellate) neurons, as well as the preference of parasubicular neurons to fire at a slightly earlier theta phase (~19° phase angle, i.e. ~7 ms, assuming an 8 Hz theta rhythm) than layer 2 pyramidal neurons, is consistent with the idea that parasubicular neurons might impose a feed-forward theta-modulated drive onto layer 2 pyramidal neurons.

Discussion

Unique features of the parasubiculum

The parasubiculum is distinct from other parahippocampal structures. The elongated shape of the parasubiculum and an almost linear arrangement of neurons differs from other (para-) hippocampal structures such as dentate gyrus, CA3, CA2, CA1, subiculum, presubiculum and medial or lateral entorhinal cortex (Amaral and Witter, 1989; Cenquizca and Swanson, 2007). Further, absence of directly-associated deep layers distinguishes the parasubiculum from the surrounding entorhinal, retrosplenial and presubicular cortices. The 'circumcurrent' axons (as defined by Burgalossi et al., 2011; Figure 1E-F) that traverse the parasubiculum and could thus establish a 'global' connectivity are also a unique feature of parasubicular anatomy. Furthermore, the parasubiculum is a preferred target of medial septal inputs and provides the major input to pyramidal neuron patches in layer 2 of medial entorhinal cortex. We observed a larger fraction of spatial and head-directional responses in the parasubiculum than in the adjacent medial entorhinal cortex (Solstad et al., 2008; Tang et al., 2014b).

Comparison to previous work

Our anatomical analysis agrees with earlier descriptions that large parts of the parasubiculum are situated between the medial entorhinal cortex and the presubiculum (Amaral and Witter, 1989; Cenquizca and Swanson, 2007). We provide evidence that the parasubiculum extends further laterally than previously thought (Van Strien et al., 2009, Boccara et al., 2010) and that this structure might lack direct association with deep layers. The idea that the parasubiculum extends dorso-laterally from the medial entorhinal cortex is based on three observations: *(i)* staining of cholinergic markers, calbindin immunoreactivity or cytochrome oxidase activity all delineate a continuous band, which extends dorso-laterally; similarly, both *(ii)* the modular structure of the 'large patches' and *(iii)* 'circumcurrent' axons extend as a continuous dorso-lateral band (Figure 1; see Burgalossi et al., 2011). Our conclusion that the parasubiculum extends dorso-laterally is strongly supported by recent high resolution mapping of gene expression in parahippocampal cortices (Ramsden et al., 2015). The authors not only observed that this dorsolateral part is different from medial entorhinal cortex, but also showed that it shares patterns of gene expression with the 'classical' medial parasubiculum

(Ramsden et al., 2015). The extent to which deep layers were assigned to the parasubiculum varies in the literature. While some studies assigned deep layers to the parasubiculum (Funahashi and Stewart, 1997; Glasgow and Chapman, 2007; Boccara et al., 2010), other work found it difficult to assign adjacent deep layers to either the presubiculum or the parasubiculum based solely on cytoarchitectonic criteria (Mulders et al., 1997). Our assessment that these deep layer neurons should not be viewed as part of the parasubiculum is based on three observations: *(i)* the shape of dorsal part of the parasubiculum, as revealed by cholinergic markers, calbindin immunoreactivity or cytochrome oxidase activity, delineates only a "superficial-layer structure" encompassing layer 1-3 (Burgalossi et al., 2011); *(ii)* we did not observe axons from the superficial parasubiculum into adjacent deep cortical layers; *(iii)* we did not observe axons from the adjacent deep cortical layers into the superficial parasubiculum. The idea that large parts of the parasubiculum lack deep layers is again supported by the gene expression analysis of Ramsden et al., 2015.

Our results agree with previous extracellular recording data that also revealed the presence of spatially-modulated neurons in the parasubiculum (Taube, 1995; Cacucci et al., 2004; Boccara et al., 2010; Burgalossi et al., 2011; Cacucci et al., 2004). The present data are also consistent with the study of Boccara et al., 2010, where the authors described grid, border and head-direction responses in the parasubiculum. Notably, the strong head-direction tuning in the parasubiculum is also consistent with previous (Fyhn et al., 2008; Wills et al., 2010) and more recent work (Giocomo et al., 2014), where sharply-tuned head-direction neurons were recorded "near" the dorsalmost border medial entorhinal cortex – hence compatible with a parasubicular origin of these signals (Figure 1; Burgalossi et al., 2011). Extracellular recordings have also identified both theta-rhythmic and non-theta-rhythmic border cells in this dorsalmost region of MEC (Solstad et al., 2008), where the parasubiculum extends in a narrow stripe above MEC (Figure 1A). We found that parasubicular border cells lock strongly to the theta rhythm, while border cells in MEC layer 2 show only weak entrainment by theta oscillations (Tang et al., 2014b). Our results show a substantial proportion of spatially irregular cells, in line with previous work (Krupic et al., 2012), which also showed a larger percentage of non-grid, spatially modulated cells in the parasubiculum compared to adjacent MEC. Spatially irregular cells could provide sufficient spatial information for coding the animal's position in space (Zhang et al., 1998; Zhang and Sejnowski, 1999).

Parasubicular discharge properties mirror those of its input structures

Parasubicular response properties match well with the properties of its inputs. Parasubicular head-direction selectivity is in line with its inputs from anterior thalamus and presubiculum (Taube, 2007). The border responses observed here are in line with subicular inputs, as numerous boundary-vector cells have been observed there (Lever et al., 2009). A prominent aspect of parasubicular activity is the strong theta-phase locking and theta rhythmicity of spike discharges. Large membrane-potential theta oscillations have also been recorded from parasubicular neurons in awake animals (Domnisoru et al., 2013). Such strong entrainment may result from the massive septal GABAergic innervation (Figure 3A,B), since GABAergic neurons in the medial septum are known to be a key theta pacemaker (Buzsáki, 2002; Hangya et al., 2009; Brandon et al., 2011; Koenig et al., 2011). Cholinergic innervation may also drive parasubicular neurons to depolarized states promoting theta oscillations (Glasgow and Chapman 2007; Glasgow and Chapman, 2013).

Does the parasubiculum provide input to the grid system?

Our data suggest a relationship between the parasubiculum and layer 2 of medial entorhinal cortex grid cells (Sargolini et al., 2006; Boccara et al., 2010). Grid cells in the medial entorhinal cortex show strong theta rhythmicity of spiking (Boccara et al., 2010). It is therefore most interesting that the strongly theta-rhythmic parasubicular neurons project selectively into layer 2 pyramidal cell patches, where neurons show strong entrainment by the theta rhythm (Ray et al., 2014) and where most grid cells might be located (Tang et al., 2014b). The discharge timing is consistent with an activation/entrainment of layer 2 pyramidal neurons by parasubicular inputs. Parasubicular neurons discharge on average at an earlier theta phase (~19° phase angle, i.e. ~7 ms) than layer 2 pyramidal neurons (Figure 7E,F). The parasubicular input to layer 2 pyramidal neurons is also remarkable, in light of the sparse excitatory connectivity within layer 2 of medial entorhinal cortex (Couey et al., 2013; Pastoll et al., 2013). Parasubicular inputs could be important for three aspects: *(i)* for imposing theta rhythmicity on grid responses, and possibly also contributing to their temporal spiking dynamics (Hafting et al., 2008, Mizuseki et al., 2009, Ray et al., 2014, Tang et al., 2014b); *(ii)* parasubicular head-directional responses could be causally related to downstream grid activity in layer 2 of MEC. Indeed, grid cells have been shown to receive head-directional

inputs and disruptions of head-direction signals also impaired grid cell firing (Bonnevie et al., 2013; Winter et al., 2015). The parasubiculum might be the source of this input, given the large-fraction of head-direction cells and the selective output to MEC layer 2 (Figure 3); (*iii*) parasubicular border activity could be needed for anchoring entorhinal layer 2 grids to environmental boundaries (Hardcastle et al., 2015). Interestingly, direct projections from border to grid cells have been recently postulated, which might be responsible for determining grid orientation, ellipticity and stability (Krupic et al., 2015; Stensola et al., 2015; Hardcastle et al., 2015; Kruge et al., 2014). The parasubiculum might be one source of border signals into the entorhinal grid system.

Functional considerations

What does the parasubiculum do? It seems likely that the parasubiculum plays a role in determining spike-timing of downstream neurons relative to theta oscillations. The massive internal connectivity of the parasubiculum by circumcurrent axons is rather unique. These axons connect along the dorso-ventral axis of the parahippocampal cortex. As different spatial scales are mapped onto the dorsoventral axis of the medial entorhinal cortex (Brun et al., 2008), we wonder whether these axons ensure that those parasubicular neurons along the dorsoventral axis signaling the same positions (at different spatial scales) fire at the same time relative to the theta cycle. Another peculiar aspect of parasubicular anatomy is the lack of strong direct hippocampal connections (van Strien et al., 2009). Together with the absence of deep layers (the recipient of CA1/subicular back-projections in the medial entorhinal cortex) and a thinner layer 1, it seems that the parasubiculum is only poorly connected to the 'trisynaptic memory loop' (reciprocal connections between the parasubiculum and postrhinal cortex could however provide an indirect pathway; Agster and Burwell, 2013). We envision the parasubiculum may function more for providing online spatial information like a pointer ('where am I?') rather than for long-term storage of information ('where was I?'). This pointer hypothesis is consistent with disruption of place cell activity (Liu et al., 2004) and working memory deficits after parasubicular lesions (Kesner and Giles, 1998).

Materials and methods

All experimental procedures were performed according to the German guidelines on animal welfare under the supervision of local ethics committees.

Brain tissue preparation

For anatomy experiments, male and female Wistar rats (150-400 g) were anesthetized by isoflurane, and then euthanized by an intraperitoneal injection of 20% urethane or sodium-pentobarbital. They were then perfused transcardially with 0.9% phosphate buffered saline solution, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). After perfusion, brains were removed from the skull and postfixed in PFA overnight. They were then transferred into a 10% sucrose solution in PB and left overnight, and subsequently immersed in 30% sucrose solution for at least 24 hours for cryoprotection. The brains were embedded in Jung Tissue Freezing Medium, and subsequently mounted on the freezing microtome to obtain 20-60 µm thick sagittal sections or tangential sections (parallel to the pial surface of the medial entorhinal cortex (MEC)). Tangential sections were obtained by removing the cerebellum, visually identifying the pial surface of the MEC (Figure 1A in Ray et al., 2014) and making a cut 3mm anterior and parallel to the pial surface of the medial entorhinal cortex. The tissue was then frozen and positioned with the pial side to the block face of the microtome.

Tissue from PV-Cre mice, expressing Cre recombinase under the parvalbumin promoter (B6;129P2-Pvalbtm1(cre)Arbr/J mice, stock no 008069, Jackson, Bar Harbor, ME, USA), was prepared using similar methods, except that the sections were cut on a standard microtome (nominal thickness 100 µm, horizontal) right after overnight fixation in PFA.

Histochemistry and immunohistochemistry

Acetylcholinesterase (AChE) activity was visualized according to previously published procedures (Ray et al., 2014). After washing brain sections in a solution containing 1 ml of 0.1 M citrate buffer (pH 6.2) and 9 ml 0.9% NaCl saline solution (CS), sections were incubated with CS containing 3 mM $CuSO_4$, 0.5 mM$K_3Fe(CN)_6$, and 1.8 mM acetylthiocholine iodide for 30 min. After rinsing in PB, reaction products were visualized by incubating the sections in PB containing 0.05% 3,3'- Diaminobenzidine (DAB) and 0.03% nickel ammonium sulfate. Immunohistochemical stainings were performed according to standard procedures. Briefly, brain sections were pre-incubated

in a blocking solution containing 0.1 M PBS, 2% Bovine Serum Albumin (BSA) and 0.5% Triton X-100 (PBS-X) for an hour at room temperature (RT). Following this, primary antibodies were diluted in a solution containing PBS-X and 1% BSA. We used primary antibodies against the calcium binding protein Calbindin (1:5000), the DNA binding neuron specific protein NeuN (1:1000) and, for the mice, against green fluorescent protein (GFP). Incubations with primary antibodies were allowed to proceed for at least 24 hours under mild shaking at 4°C in free-floating sections. Incubations with primary antibodies were followed by detection with secondary antibodies coupled to different fluorophores (Alexa 488 and 546). Secondary antibodies were diluted (1:500) in PBS-X and the reaction was allowed to proceed for two hours in the dark at RT. For multiple antibody labeling, antibodies raised in different host species were used. After the staining procedure, sections were mounted on gelatin coated glass slides with Mowiol or Vectashield mounting medium. In a subset of experiments, primary antibodies were visualized by DAB staining. For this purpose, endogenous peroxidases were first blocked by incubating brain tissue sections in methanol containing 0.3% hydrogen peroxide in the dark at RT for 30 min. The subsequent immunohistochemical procedures were performed as described above, with the exception that detection of primary antibodies was performed by biotinylated secondary antibodies and the ABC detection kit. Immunoreactivity was visualized using DAB staining.

The relative density of putative parvalbuminergic fibres in PV-Cre mice in hippocampus CA1-3, presubiculum, parasubiculum, and medial entorhinal cortex was estimated by manually outlining these four areas (Paxinos & Franklin, 2012) in epifluorescence images (2.5x) from horizontal sections (estimated depth 3mm relative to Bregma) and then measuring mean fluorescence signals in each area with the ImageJ software. For comparison between brains, these values were then normalized to the mean hippocampal value in each brain (n = 3).

Anterograde and Retrograde Neuronal Labeling

Anterograde or retrograde tracer solutions containing Biotinylated Dextrane Amine (BDA) (10% w/v; 3.000 MW or 10.000 MW) were injected in juvenile rats (~150 gr) under ketamine/xylazine anesthesia. Briefly, a small craniotomy was opened above the parasubiculum/medial entorhinal cortex. Prior to injection, the parasubiculum was localized by electrophysiological recordings, based on cortical depth, characteristic signatures of the local field potential

theta oscillations and neuronal spiking activity. Glass electrodes with a tip diameter of 10-20 μm, filled with BDA solution, were then lowered into the target region. Tracers were either pressure-injected (10 injections using positive pressure of 20 p.s.i., 10-15 s injection duration) or iontophoretically-injected (7s on/off current pulses of 1-5 mA for 15 min). After the injections, the pipettes were left in place for several minutes and slowly retracted. The craniotomies were closed by application of silicone and dental cement. The animals survived for 3-7 days before being transcardially perfused.

Viral injections and quantification of anterorgradely-traced axons
PV-Cre mice, expressing Cre recombinase under the parvalbumin promoter (B6;129P2-Pvalbtm1(cre)Arbr/J mice, stock no 008069, Jackson, Bar Harbor, ME, USA) were injected with AAV-Ef1a-dbf-hChR2(H134R)-EYFP-WPRE (serotype 1/2) roughly 6 weeks prior to perfusion. The medial septum was targeted under stereotaxic guidance: starting from the pial surface at 1 mm anterior, 0.7 mm right lateral to Bregma, a 34-gauge NanoFil™ needle (WPI, Berlin, Germany) was advanced at an angle of 10° in the coronal plane for 4200 and 4600 μm, where we injected 1 μl each (100 nl/s), waiting 5 minutes after each injection before moving the needle. The AAV virus was generously provided to us by Susanne Schoch (University of Bonn).
Fluorescence signals were normalized to dentate gyrus intensity levels and quantified. Briefly, regions of interests from horizontal sections (at a depth of ~ 3.5 mm ventral to Bregma (Paxinos & Franklin, 2012) were manually outlined and the mean fluorescence intensity for each area quantified using the ImageJ software (n = 3 mice).

Juxtacellular recordings

Juxtacellular recordings and tetrode recordings in freely moving animals were obtained in male Wistar and Long-Evans rats (150-250 g). Experimental procedures were performed, as recently described (Tang et al., 2014a; Tang et al., 2014b, Tang et al., 2015). Briefly, rats are maintained in a 12-h light/dark phase and were recorded in the dark phase. Glass pipettes with resistance 4-6 MΩ were filled with extracellular (Ringer) solution containing (in mM) NaCl 135, KCl 5.4, HEPES 5, $CaCl_2$ 1.8, and $MgCl_2$ 1 (pH = 7.2) and Neurobiotin (1-2%). Animal implantations were performed, as previously described (Tang et al., 2014a). In order to target the parasubiculum, a plastic ring was placed 0.2-0.5 mm anterior to the transverse sinus and 4.0-4.5 mm lateral to the midline. After implantation, rats were allowed to recover from the surgery and were

habituated to head fixation for 3-5 days. Rats were trained in the experimental arena (70 x 70 cm or 1 x 1 m square black box, with a white cue card on the wall) for 3-7 days Juxtacellular recordings and labeling were essentially performed as previously described (Tang et al, 2014a; Pinault, 1996). Unidentified recordings in parasubiculum were either lost before the labeling could be attempted, or the recorded neurons could not be unequivocally identified; but either pipette tracks or dendritic processes were found in the parasubiculum. After the experiment, the animals were euthanized with an overdose of ketamine, urethane or sodium-pentobarbital, and perfused transcardially with 0.1 M PB followed by 4% paraformaldehyde solution, shortly after the labeling protocol. The juxtacellular signals were amplified by the ELC-03XS amplifier (NPI Electronics) and sampled at 20 kHz by a data-acquisition interface under the control of PatchMaster 2.20 software (HEKA). The animal's location and head-direction was automatically tracked at 25 Hz by the Neuralynx video-tracking system and two head-mounted LEDs. MEC data for comparison have been published in previous papers (Ray et al., 2014; Tang et al., 2014b; Tang et al., 2015). One head-direction cell recorded and identified in the parasubiculum has been shown in a previous paper (Tang et al., 2014a).

Tetrode recordings

Tetrode recordings from parasubiculum were essentially performed as recently described (Tang et al., 2014b; Tang et al., 2015). Tetrodes were turned from 12.5 µm diameter nichrome wire (California Fine Wire Company) and gold plated to ~250 kΩ impedance. Spiking activity and local field potential were recorded at 32 kHz (Neuralynx; Digital Lynx). The local field potential was recorded from the same tetrode as single units, and referenced to a superficial silent neocortical tetrode or to the rat ground. All recordings were performed following behavioral training, as specified above for juxtacellular procedures. The animal's location and head direction was automatically tracked at 25 Hz by video tracking and head-mounted LEDs, as described above. After recordings, tetrodes were retracted from the parahippocampal areas, and multiple lesions were performed at distinct sites along the individual tetrode tracks, thereby allowing unequivocal assignment of the different tetrode tracks. Following perfusion, brains were sectioned tangentially and recording sites assigned by histology. Spikes were pre-clustered using KlustaKwik (K.D. Harris, Rutgers University) and manually using

MClust (A.D. Redish, University of Minnesota). Cluster quality was assessed by spike shape, ISI-histogram, L-ratio and isolation distance.

Neurobiotin labeling and calbindin immunohistochemistry

For histological analysis of juxtacellularly-labeled neurons, Neurobiotin was visualized with streptavidin conjugated to Alexa 546 (1:1000). Subsequently, immunohistochemistry for Calbindin was performed, as previously described (Ray et al., 2014), and visualized with Alexa Fluor 488. After fluorescence images were acquired, the Neurobiotin staining was converted into a dark DAB reaction product. Neuronal morphologies were reconstructed by computer-assisted manual reconstructions (Neurolucida).

Analysis of theta rhythmicity

Theta rhythmicity of spiking discharge was determined from the Fast Fourier Transform–based power spectrum of the spike-train autocorrelation functions of the neurons, binned at 10 ms. To measure modulation strength in the theta band (4-12 Hz), a theta power was computed, defined as the average power within 1 Hz of the maximum of the autocorrelation function in the theta rhythm (4-12 Hz). This is referred to in the paper as theta rhythmicity. Only neurons with mean firing rate > 0.5 Hz were included in the theta analysis. Statistical significance between groups was assessed by two-tailed Mann-Whitney non-parametric test with 95th confidence intervals.

Analysis of theta locking

For all neurons, we calculated the locking to theta-phase based on spiking discharge in relation to theta rhythm in the local field potential. The local field potential was zero-phase band-pass filtered (4-12 Hz) and a Hilbert transform was used to determine the instantaneous phase of the theta wave. In line with previous studies (Mizuseki et al., 2009), the theta-phase locking strength, S, and the preferred phase angle, ϕ, were defined as the modulus and argument of the Rayleigh average vector of the theta phase for all spikes. The Theta-phase locking strength value can vary between 0 (uniform distribution of spikes over the theta cycle) and 1 (all spikes have the same theta-phase). Only spikes during running (speed cutoff = 1 cm/s for juxtacellular signals, 5 cm/s for tetrode recordings) were included in the analysis. Only neurons with mean firing rate ≥ 0.5 Hz were included in the analysis. For comparison to MEC L2

data, both the analysis procedures and the juxtacellular data set correspond to our recent publications (Ray et al., 2014; Tang et al., 2014b; Tang et al., 2015).

Analysis of Spatial Modulation

The position of the rat was defined as the midpoint between two head-mounted LEDs. A running speed threshold (see above) was applied for isolating periods of rest from active movement. Color-coded firing maps were plotted. For these, space was discretized into pixels of 2.5 cm x 2.5 cm, for which the occupancy *z* of a given pixel *x* was calculated as

$$z(x) = \sum_t w(|x - x_t|)\Delta t$$

where x_t is the position of the rat at time *t*, Δt the inter-frame interval, and *w* a Gaussian smoothing kernel with σ = 5cm.

Then, the firing rate *r* was calculated as

$$r(x) = \frac{\sum_i w(|x - x_i|)}{z}$$

where x_i is the position of the rat when spike *i* was fired. The firing rate of pixels, whose occupancy *z* was less than 20 ms, was considered unreliable and not shown.

To determine the spatial periodicity of juxtacellularly recorded neurons, we determined spatial autocorrelations. The spatial autocorrelogram was based on Pearson's product moment correlation coefficient:

$$r(\tau_x, \tau_y) = \frac{n\sum f(x,y) f(x-\tau_x, y-\tau_y) - \sum f(x,y) \sum f(x-\tau_x, y-\tau_y)}{\sqrt{n\sum f(x,y)^2 - (\sum f(x,y))^2}\sqrt{n\sum f(x-\tau_x, y-\tau_y)^2 - (\sum f(x-\tau_x, y-\tau_y))^2}}$$

where, $r(\tau_x, \tau_y)$the autocorrelation between pixels or bins with spatial offset τ_x and τ_y. *f* is the image without smoothing or the firing rate map after smoothing, n is the number of overlapping pixels or bins. Autocorrelations were not estimated for lags of τ_x and τ_y, where n < 20. For spatial and head-directional analysis, both a spatial (> 50% spatial coverage) and a firing rate inclusion criterion (> 0.5 Hz) were applied. Spatial coverage was defined as the fraction of visited pixels (bins) in the arena to the total pixels.

Analysis of Spatial Information

For all neurons, we calculated the spatial information rate, I, from the spike train and rat trajectory:

$$l = \frac{1}{T}\int r(x)\log_2\frac{r(x)}{\bar{r}}o(x)dx$$

where *r(x)* and *o(x)* are the firing rate and occupancy as a function of a given pixel x in the rate map. $\bar{r}$ is the overall mean firing rate of the cell and *T* is the total duration of a recording session (Skaggs et al., 1993). A cell was determined to have a significant amount of spatial information, if the observed spatial information rate exceeded the 95th percentile of a distribution of values of I obtained by circular shuffling. Shuffling was performed by a circular time-shift of the recorded spike train relative to the rat trajectory by a random time $t' \in]0, T[$ for 1000 permutations (von Heimendahl et al., 2012; Bjerknes et al., 2014).

Analysis of Border Cells

To determine the modulation of a cell firing along a border, we determined border scores (Solstad et al., 2008). Border fields were identified from a collection of neighboring pixels having a firing rate higher than 0.3 times the maximum firing rate and covering an area of at least 100 cm (Sargolini et al., 2006). The coverage (Cm) along a wall was defined as the maximum length of a putative border field parallel to a boundary, divided by the length of the boundary. The mean firing distance (Dm) of a field was defined as the sum of the square of its distance from the boundary, weighted by the firing rate (Solstad et al., 2008). The distance from a boundary was defined as the exponential of the square of the distance in pixels from the closest boundary, normalized by half the length of the boundary. Border scores were defined as the maximum difference between Cm and Dm, divided by their sum, and ranged from -1 to +1.

Analysis of Grid Cells

Analysis of Gridness. Grid scores were calculated, as previously described (Barry et al., 2012), by taking a circular sample of the autocorrelogram, centered on, but excluding the central peak. The Pearson correlation of this circle with its rotation for 60 degrees and 120 degrees was obtained (on peak rotations) and also for rotations of 30 degrees, 90 degrees and 150 degrees (off peak rotations). Gridness was defined as the minimum difference between the on-peak rotations and off-peak rotations. To determine the grid scores, gridness was evaluated for multiple circular samples surrounding the center of the autocorrelogram with circle radii increasing in unitary steps from a minimum of 10 pixels more than the width of the radius of the central peak to the shortest edge of the autocorrelogram. The radius of the central peak was defined as the distance from the central peak to its nearest local minima in the spatial autocorrelogram. The radius of the inner circle was increased in unitary steps from the radius of the central peak to 10 pixels less than the optimal outer radius. The grid score was defined as the best score from these successive samples. Grid scores reflect both the hexagonality in a spatial field and also the regularity of the hexagon. To disentangle the effect of regularity from this index, and consider only hexagonality, we transformed the elliptically distorted hexagon into a regular hexagon and computed the grid scores (Barry et al., 2012). A linear affine transformation was applied to the elliptically distorted hexagon, to stretch it along its minor axis, until it lay on a circle, with the diameter equal to the major axis of the elliptical hexagon. The grid scores were computed on this transformed regular hexagon (Barry et al., 2012).

Analysis of head directionality

Head-direction tuning was measured as the eccentricity of the circular distribution of firing rates. For this, firing rate was binned as a function of head-direction (n = 36 bins). A cell was said to have a significant head-direction tuning, if the length of the average vector exceeded the 95th percentile of a distribution of average vector lengths calculated from shuffled data and had a Rayleigh vector length > 0.3. Data was shuffled by applying a random circular time-shift to the recorded spike train for 1000 permutations.

Classification of cells into functional categories

Cells were classified as head-direction cells, grid cells, conjunctive cells, border cells, spatially irregular cells and non-spatially modulated cells, based on their grid score, border score, spatial information and significance of head directionality according to the following criteria:

-Head-direction cells: Rayleigh vector length > 0.3 & significant head-direction tuning (Boccara et al., 2010)

-Grid cells: Grid score > 0.3 & significant spatial information.

-Border cells: Border score > 0.5 & significant spatial information (Solstad et al., 2008), or those who passed border test (Lever et al., 2009).

-Spatially irregular cells: significant spatial information (Bjerknes et al., 2014), while not passing Grid score or Border score criteria.

-Non-spatially modulated cell: no significant spatial information.

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Perspectives for Chapter I

Our work has described the parasubiculum as a clearly unique structure. This long and thin and interconnected sliver of parahippocampal cortex provides anatomically selective input to the pyramidal cell patches of layer II of the MEC. Parasubicular spatially and head directional informative cells could have a prominent role in shaping activity in the MEC both in temporal and spatial dimensions.

However, there is a lot yet to be understood about this structure and its integration in the parahippocampal circuit. As described in this Chapter, the parasubiculum is historically know to be composed of 2 parts, parasubiculum a and parasubiculum b, which express different projection patterns (Blackstad, 1956) that segregate into the dorsal and ventral streams. It is yet unknown whether these structures differ in any way functionally.

The recent discovery of time cells in the LEC (Tsao et al., 2018) would suggest that perhaps parasubiculum b, which strongly projects to LEC might be contributing with the encoding of the temporal dimension. This remains to be seen.

The fact that the parasubiculum, has grid cell activity, has strong internal connectivity, and projects to the MEC is suggestive regarding its role in the putative attractor network implementation in the brain. Further work is required to assess its functional role in the circuitry. Connectivity studies, either at the connectomics level, multiple whole cell recordings, or by analyzing neuron crosscorrelations would help elucidate further whether the parasubiculum has the necessary substrates for an attractor network or the properties to interact with one.

The most intriguing remaining questions about the parasubiculum relate to its very special connectivity with the medial septum and other regions like the amygdala. This might be related to MEC and parasubiculums role in contextual memory. In this case, parasubiculum may be involved encoding the contextual valence of a place. This idea inspired us into looking first in the parasubiculum for a neural correlates of one of the most important places in an animals life. Its home.

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CHAPTER II

Is there an online representation of Home in parahippocampal areas?

Overview

The key findings on the neurobiology of spatial navigation come mostly from an abstract approach that separates the animal from even a minimally biologically relevant context. This contrasts notably to the rich behavioral literature regarding animal navigation and use of space. Navigation is incredibly important in animals' lives as is required for multiple biologically relevant behaviors, nesting, mating, foraging, hoarding, and migrating. The overall lack of studies into the more ethological nature of how animals encode space may be hindering us from understanding key aspects of the brain.
In this chapter we make use of the fact that rats are strongly attached to their home cage in order to investigate neural correlates of the home location in spatial neural representations of the rat medial entorhinal cortex and parasubiculum.

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Home, head direction stability and grid cell distortion

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Abstract

The home is a unique location in the life of humans and animals. Numerous behavioral studies investigating homing indicate that many animals maintain an online representation of the direction of the home, a home vector. Here we placed the rat's home cage in the arena, while recording neurons in the animal's parasubiculum and medial entorhinal cortex. From a pellet hoarding paradigm it became evident that the home cage induced locomotion patterns characteristic of homing behaviors. We did not observe home-vector cells. We found that head-direction signals were unaffected by home location. However, grid cells were distorted in the presence of the home cage. While they did not globally remap, single firing fields were translocated towards the home. These effects appeared to be geometrical in nature rather than a home-specific distortion. Our work suggests that medial entorhinal cortex and parasubiculum do not contain an explicit neural representation of the home direction.

Introduction

Animals maintain and update a representation of their location in space. Such mapping abilities allow them to navigate their surroundings in search for food, safety, mates or their kin. In the case of the migratory Bar-tailed Godwit such navigation can involve a non-stop 11000 km flight from New Zealand wintering grounds to artic Siberian breeding grounds (Gill, Piersma, Hufford, Servranckx, & Riegen, 2005) a truly incredible navigational feat.

Most studies in spatial navigation and the circuitries involved disregard the contribution of variables making navigation important and necessary for the animal. The home is a unique location in the life of humans and animals. Hence numerous behavioral studies have researched homing; in pigeons (Alleva, Baldaccini, Foà, & Visalberghi, 1975), in bees is returning to the hive (Menzel et al., 2005), in salmon returning to the stream where they were born in (Neave, 1964), in bats returning to their cave from foraging their favorite tree (Tsoar et al., 2011) and many other species. Home is a safe haven for ground dwelling mammals as well, mammals organize exploratory trips from a home base (Papi, 1992), nest in burrows where they care for their offspring. Safety considerations shape rat exploratory behaviors and lab rats naturally organize their behavior around their home-cage (Whishaw, Gharbawie, Clark, & Lehmann, 2006).

From these very diverse studies a consensus emerged that patterns of navigation show remarkable and systematic variations relative to the home location. In particular, animals often show variable and complicated trajectories outgoing from the home only to return on relatively direct, straight incoming trajectories back to the home location. Such differing outgoing and incoming trajectories can be observed during foraging, but also during other navigational tasks. Indeed, some of the best evidence for path integration in the animal kingdom comes pup retrieval experiments of the Mittelstaedts (Mittelstaedt & Mittelstaedt, 1980), where the authors demonstrated path integration by the mother's tendency to return pups on a straight trajectory back to the home. In the proximity of the home startling stimuli will very often induce a short-latency escape maneuver straight back home. Thus, many animals maintain an immediately accessible online representation of the direction of the home, a representation we refer to as home vector. In our paper we ask how this home vector might be represented in the well-known neural substrates of navigation. Head Direction cells, Goal Direction cells and Grid cells have been hypothesized to sustain vectorial

navigation (Banino et al., 2018; Kubie & Fenton, 2009; Sarel, Finkelstein, Las, & Ulanovsky, 2017; Valerio & Taube, 2012).

Head Direction cells, present in several brain structures including the anterior dorsal nucleus of the thalamus, the presubiculum, the medial enthorinal cortex (MEC) and the Parasubiculum (PaS), have sharp tuning curves in relation the animals orientation in space (Tang et al., 2016; Taube, 1995; Taube, Muller, & Ranck, 1990a). The overall population encodes for each of the possible compass like directions of the animal's head in the horizontal plane. Interestingly, they are known to rotate their preference to rotation of visual cues in the environment, while maintaining internal coherence between cells (Taube et al., 1990a; Taube, Muller, & Ranck, 1990b). Some findings even point towards head direction cells also rotating in relation to local landmarks in absence of distal cues (Zugaro, Berthoz, & Wiener, 2001). In lab navigation tasks the accuracy of Head Direction cells also predicts successful navigation (Valerio & Taube, 2012).

Grid cells of the MEC and parasubiculum are known to be active in multiple spatial firing fields which tile the whole environment forming a periodic hexagonal lattice (Boccara et al., 2010; Fyhn, 2004; Hafting, Fyhn, Molden, Moser, & Moser, 2005; Tang et al., 2016). Even though recent work points towards differences in field firing rates as a way for a single cell to encode different environments and local positional information (Diehl, Hon, Leutgeb, & Leutgeb, 2017; Ismakov, Barak, Jeffery, & Derdikman, 2017), the robustness of the hexagonal grid field positions for simple environments has been studied in great detail (Stensola, Stensola, Moser, & Moser, 2015) .

We were interested in the potential role of the parasubiculum in home directed navigation. The parasubiculum is a long (3mm) and thin (300 microns) parahippocampal cortex wrapped around the medial and dorsal boundaries of the MEC. It contains both a high proportion of head directional and spatially selective cells, including grid cells and border cells (Boccara et al., 2010) , and connects selectively to pyramidal patches in layer 2 of the MEC, preceding their firing in theta-phase (Tang et al., 2016). Specifically, we ask the following questions:

(1) Do rats care about their home cage?
(2) Is the home location evident from MEC and parasubicular discharge rates?
(3) Are there home-direction cells, whose discharge is tuned to the home location?
(4) Are head-direction signals altered or distorted by the home location?
(5) Are grid cell signals altered or distorted by the home location?

Our data indicate that the home-cage alters rat locomotion patterns in a way that is characteristic for homing behaviors. We did not observe home-direction cells and found that head-direction signals are not affected by the home location. Grid cell signals were locally altered by the home cage location, but the effects appeared to be more geometrical in nature rather than a home-specific distortion of spatial discharge.

Results

In our study we addressed the question how neurons in the rat medial entorhinal cortex and the rat parasubiculum represent the home location. To this end we recorded from freely moving male Long Evans rats (n=6) using tetrodes. Rats were familiarized for two weeks with a 1m x 1m squared environment with round edges containing a principal cue card multiple irregularly shaped sub-cues, both on the walls or the floor of the arena (Figure1A). During the same period, we housed rats in custom-modified home-cage modified with 2 side doors (Figure1A). To assess if the home cage affected the rat's spatial behavior we studied rats performing pellet hoarding. During pellet hoarding (Figure 1; Wolfe, 1939) rats forage for food pellets and perform high-speed return vectors towards their safe home location. In our setting (Figure 1A) rats foraged large food pellets in a 1 meter arena in the presence of their home cage. Without specific prior training rats foraged these pellets and cached them in their home cage. The behavior was stereotypical, consisting of high speed return trips as previously described in the literature (Maaswinkel & Whishaw, 1999; Winter, Blankenship, & Mehlman, 2018)(Figure 1 B). Running speed was visibly lower during exploratory trips away from their home (Figure 1 C). As has been described in the literature (Tchernichovski, Benjamini, & Golani, 1998; Wallace, Martin, & Winter, 2008; Winter et al., 2018) incoming trips (Figure 1D) consisted of higher speeds ($p<0.001$, Krustal-Wallis) than outgoing (Figure 1E). Thus, the home cage in the arena setting greatly altered the rat's locomotion patterns and divided them into irregular, slow exploratory outgoing trajectories and relatively straight, fast return trips to the home cage. These observations suggest that the home cage can induce homing behaviors.

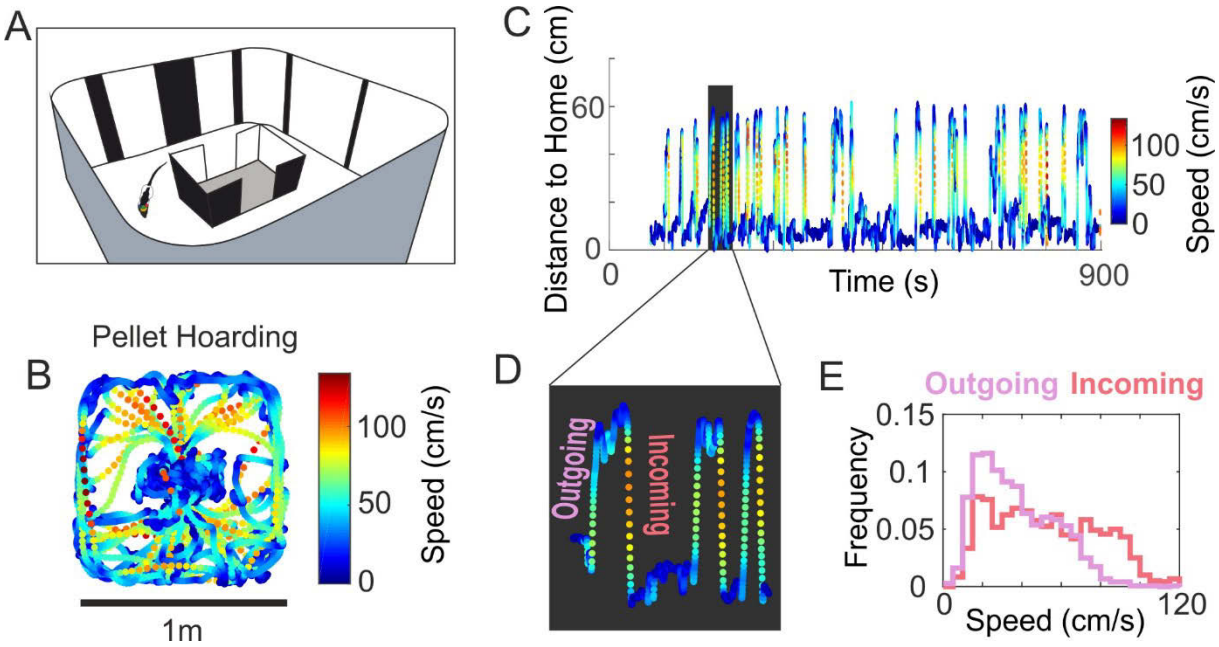


Figure 1. In a pellet hoarding paradigm the home cage induces differential outgoing and incoming locomotion reflecting homing behavior.

A) Top: Schematics of the environment with the home cage in the center; the animal's home cage was modified such that not only the lid could be removed, but that also two gaps in the sides of the cage could be opened. Walls are covered in complex cues. Bottom: We recorded 12-20 minute sessions without removing the rat form the arena.

B) Speed profile. Trajectory of the animal in the environment are color-coded according to running speed while the rat is hoarding pellets in the home in the center of the arena.

C) Rat performed exploratory trips from the home. Speed is color coded. Distance to the home plot against time for a rat performing pellet hoarding behavior.

D) Magnified view of three single foraging trips in C.

E) Histograms of proportions of speeds for outgoing (pink) and homecoming (red) exploratory trips. ($p<0.001$, Krustal-Wallis)

We wondered how the presence of the home cage would affect head direction cells and grid cells, and whether we could find any home vector representation informing the rat about how to get back home. Once the tetrodes reached parahippocampal structures we recorded single units in the parasubiculum, MEC and medial MEC **(Ray, Burgalossi, Brecht, & Naumann, 2017)** in several sessions while the animal was foraging for small treats. We recorded sessions of 12-25 minutes starting with an open field recording and following with sessions where we placed the rat's own home-cage in different places in the arena. In order not to disturb the familiarity of the rat with the arena, we did not remove or disorient the rat. This was facilitated by the use of a wireless logger system for recording and allowed us to test exclusively for the effects of locally altering the internal geometry of the environment. We recorded (n=500) cells in the parasubiculum and MEC, which we classified into Pure head direction Cells (n=90), Pure grid cells (n=35), conjunctive grid cells (n=50) and Rest (n=325). We analyzed whether the presence of the home

resulted in pure or conjunctive representations of the direction towards the home and whether it affects the encoding of the environment by head direction cells and grid cells.

Head direction activity is not affected by home cage location

As the first step of our analysis of neural responses we assessed how the home cage affected head direction signals (Figure 2). We recorded (n=68) head direction cells in the PaS/MEC with the presence of the home in the center of the environment or the home rotated and translated to the edge of the environment (n=20; Figure 2A) and found that pure head direction cells remained stable during both the introduction and the translation of the animals home (Figure 2B/C). Differences in Rayleigh vector length of head direction in comparison to the open field condition were centered on zero showing no bias towards an increase or decrease of head directional coding (Figure 2D). Head direction cells also maintained their angular preference. This is obvious from the cumulative distribution of angle differences between home cage and open field condition, which is narrowly centered at 0 degrees (Figure 2E). Overall, there was a strong correlation between the angular rate distributions with and without the home (Figure2D). The head directionality of conjunctive grid cells was also unaffected by the presence of the home (Supplementary Figure 1), both in angle preference (Supplementary Figure 1 D) and vector length (Supplementary Figure 1 E). All in all we conclude that the presence of the home does not affect head direction tuning properties.

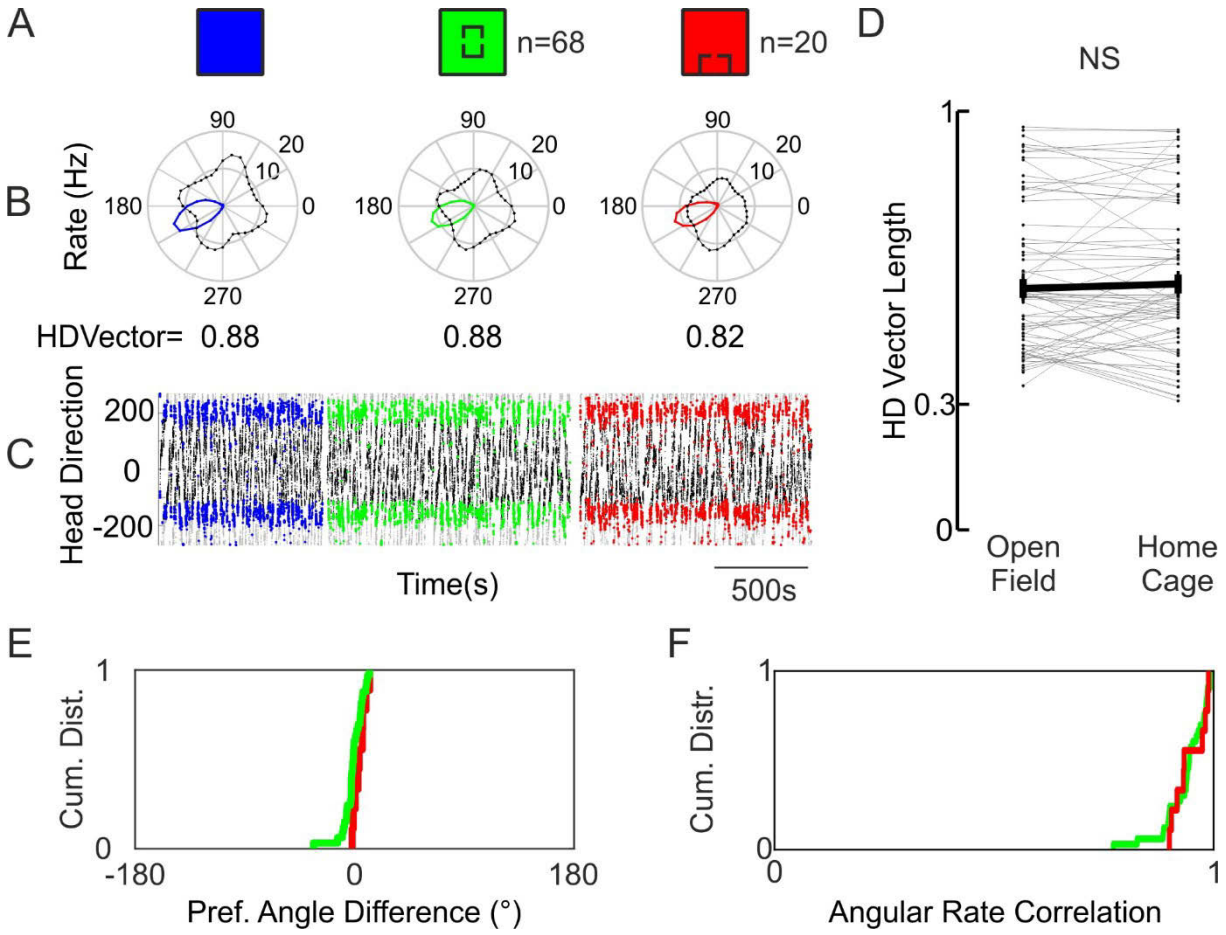


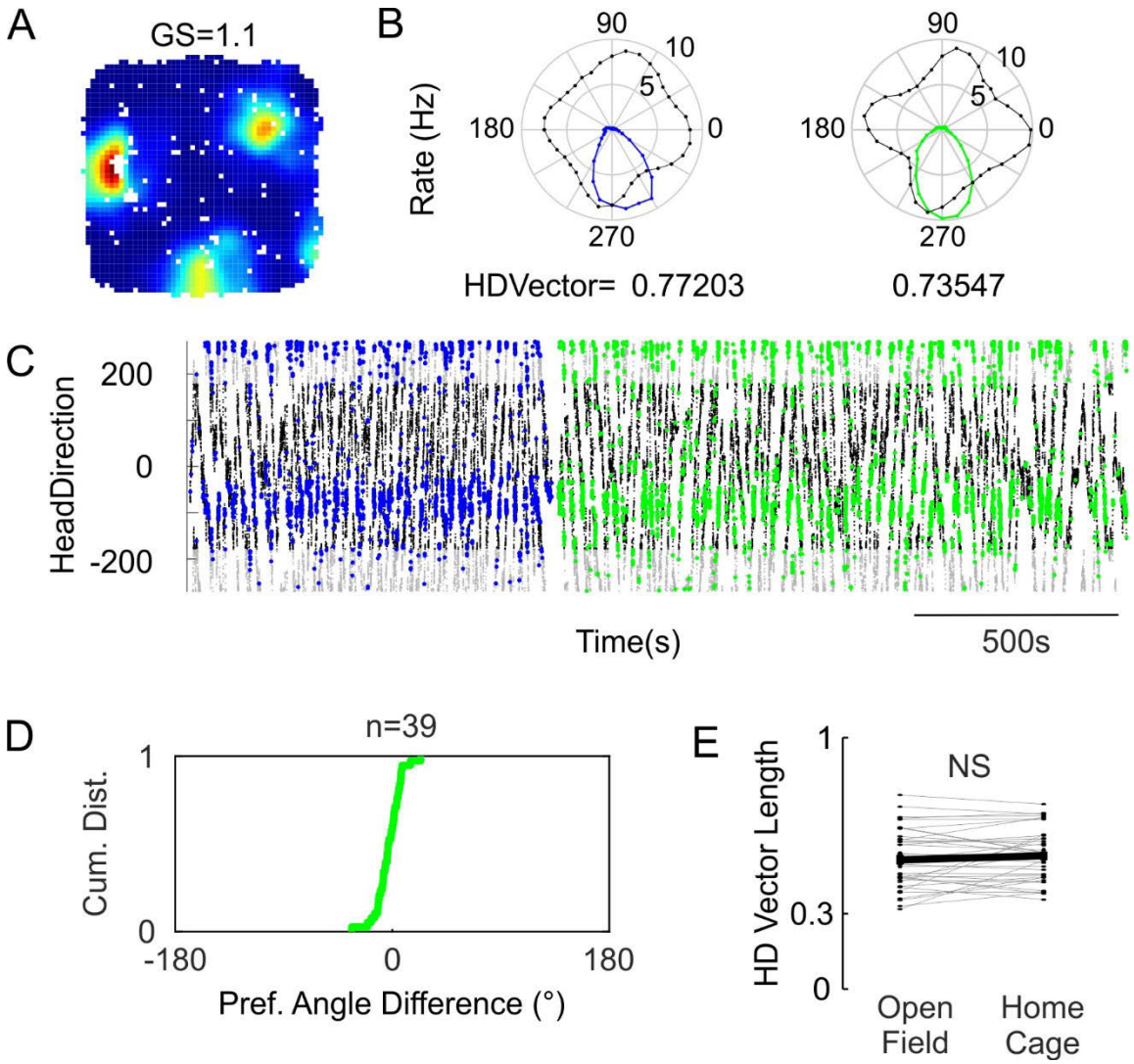
Figure 2. Head direction discharge is not affected by home cage location.

A) Schematic of the conditions depicted for a HD cell, Open Field (Blue), Home Center (green), Home Moved (Red). B) Head direction rate polar plots (rate in corresponding color, angular occupancy in black). C) Spikes of the HD cell are clearly preferring a stable head direction. Black and grey, Head direction of the animal in time (duplicated for visualization). Spikes are plotted on top in the corresponding RGB color scheme.

D) Head Direction Rayleigh vector lengths are not affected by the presence of the home cage. (KS Normality test, p=0.34, t-test, p=0.96, N=68)

E) Cumulative frequency function of the distributions of differences between preferred angle in the Open Field and in each Home Condition. Note that the inflection point is at 0 and the very steep slope. (KS Normality Test for Angle Difference, p=0.6993)

F) Correlation between the Angular Rates of both home conditions in relation to the open field. Cumulative frequency graph shows that correlations are distributed close to 1. Black lines represent individual cells. Colored lines represent the mean and SE for each condition.



Supplementary Figure 1. Head direction discharge of grid cells is not affected by the home cage.

A) Grid cell in the open field B) Head direction polar plots show a consistent head directionality between sessions: Open Field (blue) and home center (green) conditions. C) Spikes (blue and green) plotted on top of head direction variable show the consistency of this preference over time.

D) Cumulative distribution of Preferred Angle differences in conjunctive grid cells. . (KS Normality Test, p=0.26, N=39)

E) Head Direction Rayleigh vector lengths are not affected by the presence of the home cage. (KS Normality Test, p=0.036, Signed-Rank Test, p=0.856, N=39)

Absence of explicit home vector cells

As a second step, we assessed if the home cage could induce an explicit home vector representation, i.e. neural discharges tuned to the direction of the home cage (Figure 3). We therefore computed home direction as schematized

in Figure 3A, where a home direction of 0 corresponds to when the animal is facing the home (Figure 3A bottom). We performed this computation fictively in the open field in absence of home cage (blue Figure 3B top, relative to where the center of the home cage would later appear) and relative to the real home cage (green Figure 3B top). Differences between the results of these two computations could be indicative of a home vector representation. In Figure 3C-D we show data from one of the non-grid and non-Head Direction cells with the strongest home direction tuning in terms of vector length. As shown in the polar rate plot and in the time resolved distribution of spikes in the home direction space (Figure 3B middle and bottom, respectively), even in this cell there is no strong home direction tuning (Vector Length=0.28). We found that in the general population of cells the distribution of Rayleigh vector for the resulting angular firing rates remains mostly below cut-off level used for similar variables like head directionality vector length (0.35 in our case; Figure 3E). We then investigated home direction tuning in both grid cell and head direction populations recorded in MEC and PaS to test for a representation of home direction in a conjunctive way. This analysis did not reveal any home direction tuning. Figure 3F-G shows an example corresponding to a head direction cell with a very strong head direction vector (Figure 3F) and a very weak home vector (Figure3G). We compared home direction vectors and Head Direction vectors of the HD cell population and found a large difference in their distribution strongly favoring Head Directionality (Figure 3H). Besides presenting very low Home Direction vectors, the presence of the home did not affect the encoding of home vectors in the HD cell population (Figure 3I). Similarly, grid cells did not represent home direction vectors. For the grid cell population we found very short home vector lengths and no effect of the introduction of the home in the environment (Figure 3K). These data indicate that there is no home vector tuning in the entorhinal and parasubicular cells.

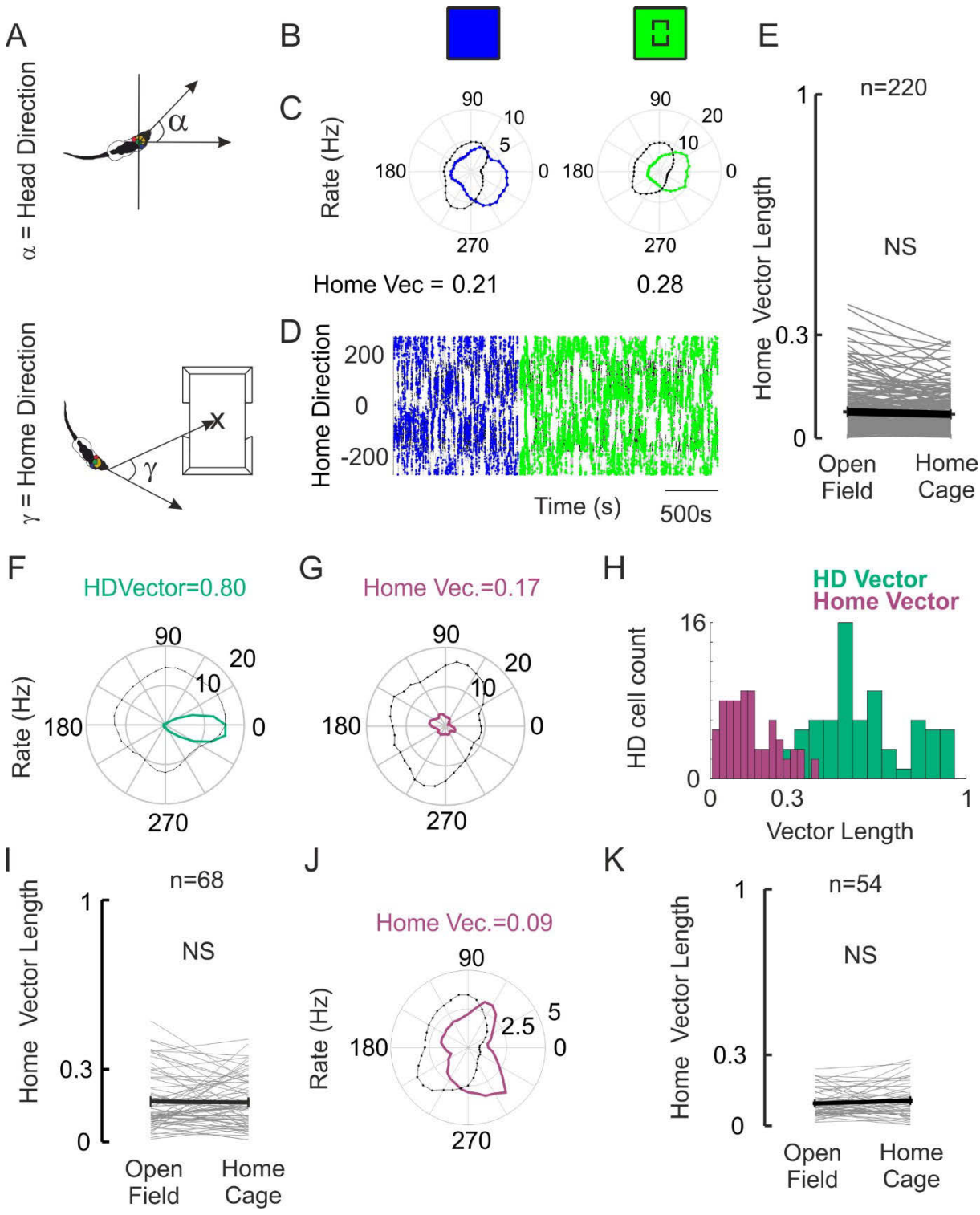

A
α = Head Direction
α
γ = Home Direction
γ
B
C
Rate (Hz)
90
10
5
180
0
270
Home Vec = 0.21
90
20
10
180
0
270
0.28
D
Home Direction
200
0
-200
Time (s)
500s
E
n=220
NS
Home Vector Length
1
0.3
0
Open Field
Home Cage
F
HDVector=0.80
Rate (Hz)
90
20
10
180
0
270
G
Home Vec.=0.17
90
20
10
180
0
270
H
HD Vector
Home Vector
HD cell count
16
0
0
0.3
1
Vector Length
I
n=68
NS
Home Vector Length
1
0.3
0
Open Field
Home Cage
J
Home Vec.=0.09
Rate (Hz)
90
5
2.5
180
0
270
K
n=54
NS
Home Vector Length
1
0.3
0
Open Field
Home Cage

Figure 3. The home cage does not induce home vector discharges.

A) Top: Representation of Head Direction Angle. Bottom: Representation of Home Direction angle. Note that in this case corresponds to the angle between the head direction vector and the vector pointing from the head of the animal to the center of the home(x). Hence, the animal facing the home retrieves a value of 0 and the animal running away from the home results in a value of 180 or -180

B) Schematic of the conditions depicted for a non-grid and non-HD cell , Open Field (Blue), Home Center (green) C) Polar Plots of Home Direction Rates (blue and green according to B, showing no clear Home Direction vectors). D) Plot of the Home Directionality of the spikes for this cell, shows no clear home direction preference.

E) Distribution of fictive Home Direction Vector Lengths in the Open Field (calculated to the center of the arena) and the corresponding vector lengths once the home cage is placed at the center of the arena. Mean and SE are depicted in black showing no significant increase in vector length. (KS Normality, p=0.0037, Signed-Rank Test, p=0.21, N=220)

F). The head direction vector of one Head Direction cell.

G) Note the lack of clear home vector tuning curve for the same cell as in F.

H) The distribution for all pure HD cells of Home Vector Lengths is much smaller than for head direction. I) Home vector lengths of pure HD cells did not change with the presence of the home. (KS Normality, p=0.367, t-test, p=0.560, N=68)

J) Home Direction polar plot for pure Grid cell, showing lack of home vector. J

K) Home Vector Lengths for all Grid cells are very low and did not change with the presence of the home. (KS Normality, p=0.780, t-test, p=0.250, N=54)

Globally stable grid cells translocate single fields towards the home cage

Thirdly, we assessed how the home cage affects positional signals and in particular grid cell discharges (Figure 4). Already implied by the stability of the head direction system to home cage insertion, the animal must remain familiar with the global environment. In line with the lack of effect on head direction cells, grid cells did not globally remap because of home cage insertion. We observed, however, that a proportion (60%) of grid cells recorded in both the parasubiculum and the medial MEC in the presence of the home cage did alter their discharge patterns. Figure 4A-B shows a grid cell recorded in the MEC for which the introduction of the home in the center of the arena retained the global representation but resulted in a local shift of a single firing field towards the home. It can be clearly observed how one central field in the green Home Cage in the center condition is shifted towards the position of the Home Cage. This is visible both at the level of spike positions (Figure 4B Top) where the green spikes are clearly shifted in position or in Figure 4B Bottom, where a composite normalized rate map using the RGB color scheme also clearly depicts the displacement of the central grid field in the green condition. Figure 4C presents two further examples of grid cells modifying their activity by translocating fields towards the location of the home-cage. We wondered if these shifts affect rates of grid cells inside the home cage. In order to be able to compare grid cells with distinct firing rates, and phases, we looked at rates of individual cells normalized to the average

rate of the cell. If we compare normalized rates of the grid cells inside the home, with the normalized rates in the equivalent area of space during the open field session, we note a significant increase in these rates in the population (Figure 4D). This change is also evident in the change in the profile of normalized rates with the Euclidian distance to the center of the home (Figure 4E) and in the spatial average of the normalized cells (Figure 4F). Spatially averaging the normalized rate maps of all grid cells in the open field and in the home center condition shows a local spatial increase in the firing rate, which can be further visualized by calculating the difference between the averages (Figure 4F-right). However, not all grid cells increased their normalized rate inside the home, in a smaller proportion it decreased (Figure 4G). We used this *ad hoc* classification to disentangle possible differences due to the original configuration of the grid. Once we separated these two populations and performed the spatial averages, and euclidian average (Figure 4H) it became clear that positively modulating cells contribute to this effect. This result is of course tautological, but we observed in addition, that the average of these cells in the open field session had a low rate in the area where the home was to be placed originally. These observations indicate that introducing the home cage boosted firing in cells that did initially had no grid firing node at the position where the Home Cage would be placed (Figure 4H Top). On the other hand, negatively modulating cells, tend to have an original field in the center of the arena, where the home will be placed (Figure 4H Bottom). Collectively, these observation show that the grid cells, which do not have a firing node in the home location, alter their firing patterns. Specifically it appears that a firing nodes close to the home cage get 'sucked' into home location.

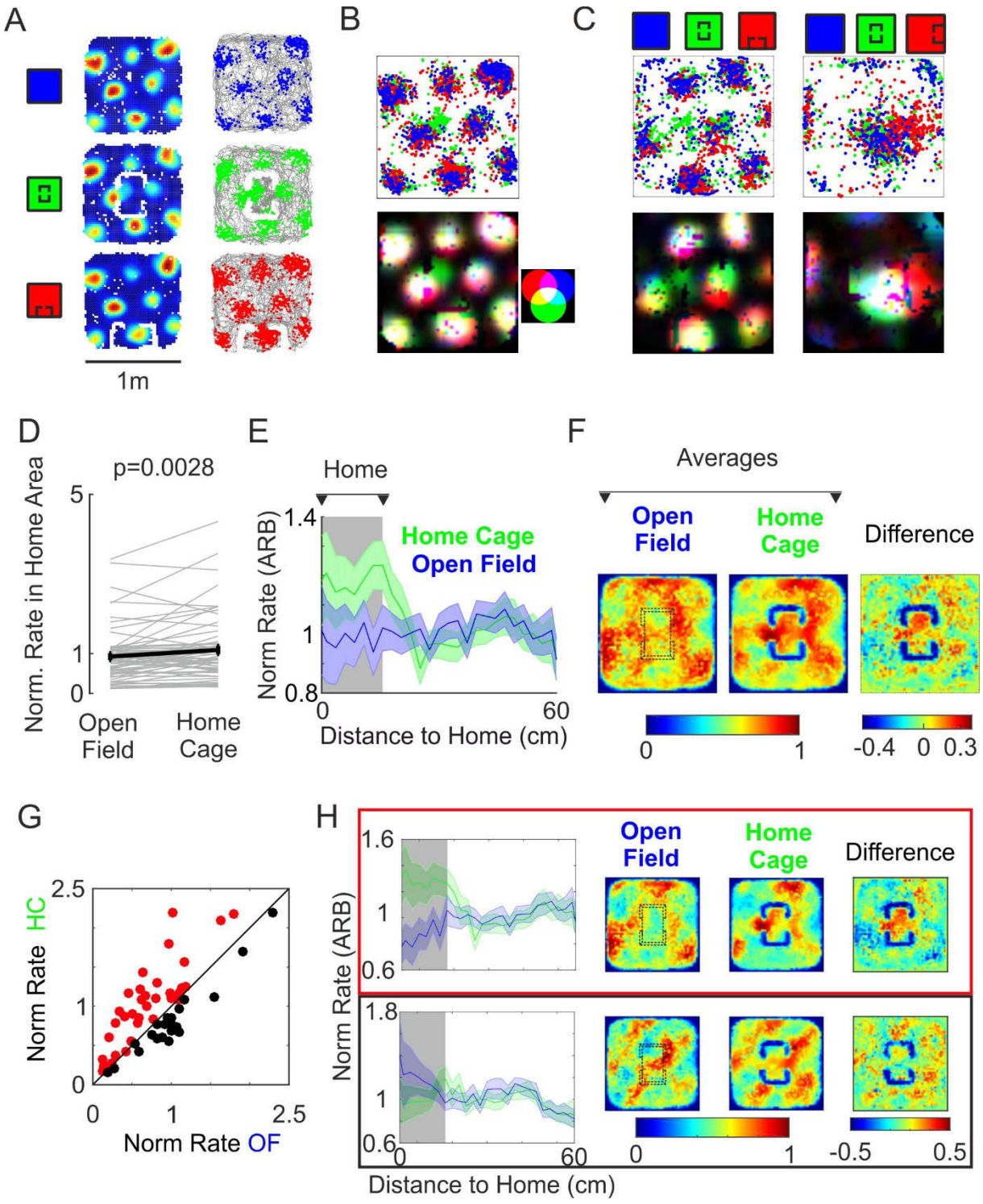

A
B
C
1m
D
p=0.0028
Norm. Rate in Home Area
5
1
0
Open Field
Home Cage
E
Home
Norm Rate (ARB)
1.4
1
0.8
Home Cage
Open Field
0
60
Distance to Home (cm)
F
Averages
Open Field
Home Cage
Difference
0
1
-0.4 0 0.3
G
Norm Rate HC
2.5
1
0
0
1
2.5
Norm Rate OF
H
Norm Rate (ARB)
1.6
1
0.6
1.8
1
0.6
Open Field
Home Cage
Difference
0
60
0
1
-0.5
0.5
Distance to Home (cm)

Figure 4. Single Firing Fields of Grid Cells shift towards Home Cage Location.

A) Grid Cell under 3 conditions, Open Field, Home Center and Home Moved (Blue, Green, Red respectively). Left: Normalized Rate Maps. Right: Spikes (RGB) superimposed on the rat trajectory (grey). B) Composite plot of the Spike Positions for the three conditions of the cell in A. Note the change in positions for Home Center spikes. Bottom; Composite Rate Map, each rate map is normalized and assigned the corresponding channel in an RGB image. Side Panel demonstrates the color compositions for the different RGB mixtures. (Cell Recorded in dorsal medial MEC)

C) Two parasubicular Grid Cells, under the same 3 conditions as in B. Note how single fields move towards the position of the home. D) Normalized firing rate increases (ARB: arbitrary units) in a region of the arena corresponding to the location of the home in comparison to the same area in the open field condition (KS Normality, p=0.280, t-test, p=0.0029, N=51). E) The increase in normalized rate is evident in the Euclidian profile to the center of the home. Solid line corresponds to mean normalized rate, and shaded area correspond to the SEM. Green: Home center. Blue: Open Field. F) The same effect is evident in the spatial averages of the normalized rates. Spatial Averages of normalized rate maps for all cells in the Open Field (Left) and Home Center (center). On the right the difference between these two average maps is shown G) Brute force split between cells up-modulating their normalized firing rate in the home area (Red, n=32) and down-modulating (Black. n=19) H) Top: Same scheme as F) for up-modulating cells from (G), with the inclusion of the Euclidian profile to the left. Bottom: Same scheme for down-modulating cells from (G), with the inclusion of the Euclidian profile to the left.

To quantify the local grid cell changes we performed a sliding window correlation analysis **(Wernle et al., 2018)** between the normalized spatial rate maps in the open field and home presence conditions. For any given pixel in the arena we selected a surrounding squared region (of dimension similar to the cells average grid spacing). This region matches spatially for both conditions (Figure 5A, boxes in white dotted lines). For each region the cell's firing rates were correlated between Open Field and Home conditions. The result is a heat-map of local correlation values between the different conditions. In this example, the presence of the home reduced locally the correlation of the grid (Figure 5A, right). We averaged these correlation maps for all grid cells and found that these show a mean local decrease in correlation corresponding to the location of the home in the center (Figure 5B left). Notably, the decrease in mean correlation follows the home, when we moved it to the side of the arena (Figure 5B, right). The decorrelation of grid cell activity appears to be local and related to the position of the home in the environment. This analysis reinforces the idea that presence of the home resulted in a change in the local activity of the grid while not affecting the global encoding of space. We were curious whether this effect might be driven or intensified by the positive valence of the home or if grid cells are just encoding for the changes in local geometry introduced with the home. To disentangle this we performed additional controls with a cardboard box with equal dimensions to the home, yet without both the familiarity and the social valence of the home. We found, however that the correlated activity of grid cells in the presence of the box drove a similar local change with respect to the open field as had the home (Figure 5C Left). At the same time activity in

the presence of the box correlated strongly with the activity in the presence of the home (Figure 5C Right). Hence, we believe that the effect seen of grid cells is indeed related to the change in the internal structure of the environment. This could be related to, just the presence of an "object" in the environment. To dissect this further we tested whether tall objects, which do not alter the space substantially, have a similar local effect on grid cells. We found that the simple presence of an object does not decrease the correlation of grids around it (Figure 5D). These results point towards the requirement of a change in the internal structure of the environment which would considerably affect the trajectories available to the rat. We wondered if single tall objects do not produce the same effect, perhaps because they minimally affect the availability of paths. Pursuing this idea further we presented the animals with new internal structures inside the environment. We used an open corridor of 70 x 10 cm, which we could place in the arena in different orientations. The presence of a corridor inside an open field grossly changes the availability of paths in the same familiar environment. Our prediction would be that grid cells would care about the position of this corridor and modify their firing fields to better represent this salient path. Introducing such corridor revealed big shifts in grid fields towards the corridor in some, but not all conditions (Figure 5E, moving field highlighted in pink). This example further demonstrates the effect of internal geometry in purely local grid cell activity.

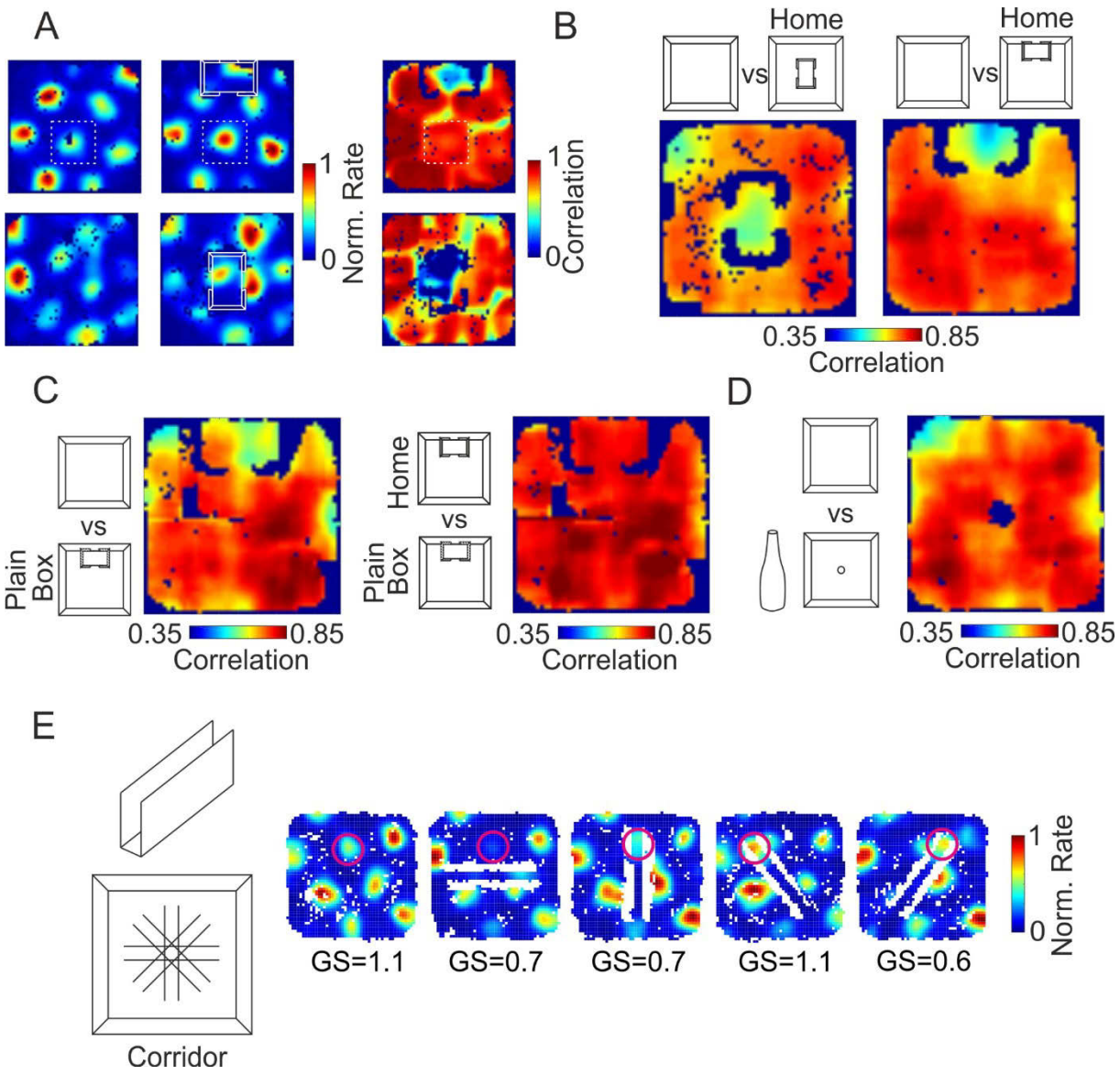


Figure 5. Effect of Home on grid cell population is driven by geometry.

A) Sliding window correlation analysis between conditions. Top: Example of normalized rate maps of a Grid Cell in the Open Field (left) and Home North (center) condition, and the corresponding sliding window correlation between these two (right). Bottom: Same as top but for another cell compared to the Home Center condition.

B) Sliding window correlation analysis for all Grid cells in both conditions. Note the lower average local correlation in the position of the home for both cases.

C) Sliding window correlation analysis comparing the Home vs a cardboard box with similar dimensions, showing that the effect is related to geometry not valence of the home. Left: the comparison between the Open Field and the Cardboard box is shown. Right: High correlation comparison between the Home and the cardboard box.

D) Sliding window correlation analysis between the Open Field and a Tall Object in the center of the arena did not produce low correlations near the object.

E) The presence of a linear corridor in the arena in different angles produced shifts of grid fields in some of the conditions further demonstrating the strong effect of internal geometry. Moving field highlighted in pink.

Discussion

The home-cage is a highly relevant location for the rat. Consistent with this idea the home cage was capable of inducing characteristics of natural homing behavior. Even though, the parasubiculum and the MEC are causally linked to the expression of spatial navigation, and contain a panoply of cells encoding variables linked to space and orientation, we did not find an explicit firing rate representation of a home vector. Specifically, in the same quality of needle like encoding as the goal direction cells found by Sarel et al in the hippocampus of freely flying bats with respect to a home-like platform (Sarel et al., 2017).

We found that both head direction cells and the head directional component of conjunctive grid cells are not affected by the presence of the home. The preferred head direction is retained, even if the home is translated to one of the sides of the arena breaking symmetry even further. This points to head direction cells and grid cells maintaining their encoding of global familiar environment, i.e., there is no remapping because of the appearance of the home. The fact that the rat was never removed from the arena or disoriented between sessions probably explains the consistency of the encoding for the global environment. Many of the traditional cue card rotation experiments, where head direction realignment was found were performed in cue-deprived conditions and with a step of disorientation of the animal between sessions.

Even though earlier work had shown that grid cell macro crystalline hexagonal patterns break in complex shaped environments like the hairpin maze, and more recent studies have shown global distortions are possible when exposing the animal to irregular shaped environments or changing external boundaries (Krupic, Bauza, Burton, Barry, & O'Keefe, 2015; Krupic, Bauza, Burton, & O'Keefe, 2018), we are just beginning to understand how the structure of the environment affects single firing fields and whether these distortions might contribute encoding more intricate environments.

In similar manner a study by Wernle and collaborators shows a much faster change in the grid pattern of cells encoding two environments separated by a wall, before and after said wall is removed (Wernle et al., 2018). Grid cells showed a tendency towards a more global representation by stitching up the previous two global grid patterns, into a meta-global grid pattern. This intriguing study points to a strong role of borders in the establishment of the grid pattern. However, in this case it is hard to disentangle what might be the single contribution of local structures, since the overall global environment is changed in shape and size.

Part of our contribution lies in testing what are the micro level effects of internal structures of the environment (in our case the rat's home) on grid fields of well learnt, familiar environments without disturbing the global environment itself. In line with the head direction, grid cells did not globally remap with the presence of the home. In all cases they retained their previous phase, orientation and spacing. However, we did find that grid cells are far from being a perfect crystal projected on to the arena. We observed shifts of local firing fields towards the position of the home. This phenomenon manifested itself by a local decrease in correlation of the rate maps of the grid cells in the vicinity of the home, while global correlation of the grids is preserved.

The relocation of grid fields due to changes in the internal structure of the environment may have a critical role in vector based navigation and route planning. The most recent interpretation of the role of grid cells in spatial navigation relates to the possibility of using grid populations to directly calculate the shortest path between two points (Banino et al., 2018; Kubie & Fenton, 2009). Changes in the internal structure of the environment restructure the availability of paths in the environment. Some paths need to be better represented, while some others are now impossible. The hexagonal grid cell in the open field, might be the best way of encoding all possible segments, which for example includes all possible directions. As has been described, when the same cells are recorded in a hairpin maze, grid cells become linearized inside each transect and repeating for different pins of the maze and at the same time becomes dependent on the direction of travel. This restructuring of the grids is in line with the restructuring of possible paths in the arena, the only vectors that can be calculated are ones going back and forth through each hairpin. When rats were trained to perform a zig-zag in an open field environment, behaviorally mimicking the hairpin like behavior, the gridness of the cells were once again present, given that all the possible paths were again available (Derdikman et al., 2009).

The shifts found for grid cells together with the up-modulation of firing rates in the home point towards an increased overlap in the grid population firing in the position of the home. This could allow for a more resolved encoding of that area of the environment. Our results are in-line with recent work showing the adaptability of grid cells to changes to their global environment (Krupic et al., 2018; Wernle et al., 2018). In addition, we have shown that grid cells also flexibly encode local internal changes in the environment, pointing towards their role in encoding more naturalistic environments and suggesting a clear

hypothesis towards their role in allowing vectorial navigation in complex environments.
While our study confirms on a behavioral level that the home cage is a unique location of rats, we have not been able to decipher a neural signature of what makes home a special place. We wonder, if such a 'neural home signature' exists in the corticohippocampal system or if subcortical circuits provide the animal with this information.

Materials and methods

All experimental procedures were performed according to the German guidelines on animal welfare under the supervision of local ethics committees.

Subjects

We obtained data from 6 male Long Evans rats (~300g) using chronic extracellular recordings.

Tetrode recordings

Tetrode recordings from parasubiculum and MEC were performed, as recently described (Tang et al., 2016).
Tetrodes were turned from 12.5 µm diameter nichrome wire (California Fine Wire Company) and gold plated to 250-300 kΩ impedance. In order to identify tetrodes in the complex anatomy of the Parasubiculum and MEC, tetrodes we stained with fluorescent tracers DiI and DiD (ThermoFisher, Scientific) before implantation.
Rats were chronically implanted with 32 channel Harlan-8 Drives (Neuralynx) with independently movable tetrodes.
Spiking activity and local field potential were recorded at 32 kHz (Neuralynx; Digital Lynx) or at 32 kHz using the wire free RatLogger-32 from Deuteron Technologies Ltd. All recordings were done freely-moving during behavioral tasks. The animal's location and head direction was automatically tracked at 25 Hz by video tracking with a colored camera using Red-Blue head-mounted LEDs or Red-Blue colored plastic targets. After recordings, the animals were transcardially perfused. Spikes were detected and clustered using Kilosort (Pachitariu, Steinmetz, Kadir, Carandini, & Harris, 2016) and manually curated based on PCA overlap and refractory period violations using the visualization toolbox Phy.

Behavioral Procedures

After surgery animals were adapted for two weeks to a modified home-cage with 2 side doors. Concurrently, the animals were put under food restriction up to achieving 80% of their ad-libitum feeding body weight, and adapted to foraging small chocolate treats while familiarized with a cue rich 1x1m arena under well-lit conditions.

Once the tetrode reached the parasubiculum and MEC (based on the presence of strong theta), we recorded from 2 to 8 sessions per day of 12-18 minute each, while the animal explored the same familiar arena without removing or disorienting the rat between sessions. In between each session we introduced, or displaced the home-cage of the animal or additional control objects (Bottle, Plain Box, and Corridor).

Hoarding

In n=5 rats we performed hoarding behavioral tests. For these we positioned the home-cage in the center of the arena, and instead of randomly dispersing chocolate treats, we dispersed standard food pellets outside the rats home-cage. Food deprived rats, retrieved these pellets and horded them inside the home cage without any specific training. Rat's hoarded up to 80 pellets in 20 minutes.

Histology

After perfusion, the brain was post-fixed in PFA 4% for 12-18hrs. The brain was then sectioned tangentially using the methods described in **(Lauer, Schneeweiß, Brecht, & Ray, 2018)** and recording sites assigned by histology using Inmunohistochemistry of Calbindin to correctly assign the parasubiculum and MEC recordings.

We did not see significant differences in the populations and pooled cells from parasubiculum, and MEC.

Analysis of Spatial Modulation

The position of the rat was defined as the midpoint between two head-mounted LEDs or colored targets. A running speed threshold (of 5cm/s) was applied for isolating periods of rest from active movement. Color-coded firing maps were plotted. For these, space was discretized into pixels of 2 cm x 2 cm, for which the occupancy z of a given pixel x was calculated as

$$z(x) = \sum_t w(|x - x_t|)\Delta t$$

where x_t is the position of the rat at time *t,* Δt the inter-frame interval, and w a Gaussian smoothing kernel with σ = 5cm.

Then, the firing rate r was calculated as

$$r(x) = \frac{\sum_i w(|x - x_i|)}{z}$$

where x_i is the position of the rat when spike *i* was fired. The firing rate of pixels, whose occupancy *z* was less than 20 ms, was considered unreliable and not shown.

For spatial and head-directional analysis, both a spatial (> 50% spatial coverage) and a firing rate inclusion criterion (> 0.5 Hz) were applied. Spatial coverage was defined as the fraction of visited pixels (bins) in the arena to the total pixels.

Analysis of Spatial Information

For all neurons, we calculated the spatial information rate, *I*, from the spike train and rat trajectory:

$$l = \frac{1}{T} \int r(x) \log_2 \frac{r(x)}{\bar{r}} o(x) dx$$

where *r(x)* and *o(x)* are the firing rate and occupancy as a function of a given pixel x in the rate map. $\bar{r}$ is the overall mean firing rate of the cell and *T* is the total duration of a recording session (Skaggs, McNaughton, & Gothard, 1993). A cell was determined to have a significant amount of spatial information, if the observed spatial information rate exceeded the 95th percentile of a distribution of values of I obtained by circular shuffling. Shuffling was performed by a circular time-shift of the recorded spike train relative to the rat trajectory by a random time for 1000 permutations.

Analysis of Grid Cells

Analysis of Gridness. Grid scores were calculated, using publically available codes from the Derdikman Lab's recent publication (Ismakov et al., 2017). By taking the autocorrelogram, centered on, but excluding the central peak. The Pearson correlation of the autocorrelogram with its rotation for 60 degrees and 120 degrees was obtained (on peak rotations) and also for rotations of 30 degrees, 90 degrees and 150 degrees (off peak rotations). Gridness was defined as the minimum difference between the on-peak rotations and off-peak rotations.

Analysis of head directionality

Head-direction tuning was measured as the eccentricity of the circular distribution of firing rates. For this, firing rate was binned as a function of head-direction (n = 36 bins). A cell was said to have a significant head-direction

tuning, if the length of the average vector exceeded the 95th percentile of a distribution of average vector lengths calculated from shuffled data and had a Rayleigh vector length > 0.35. Data was shuffled by applying a random circular time-shift to the recorded spike train for 1000 permutations.
We studied the head directional properties across subsequent conditions with the presence of the home by analyzing changes in both the angle of the Rayleigh vector or the modulus of the vector.

Home Direction Analysis

Home Direction is calculated as in Figure 3A, as the angle the rat would have to turn its head to face in the direction of the home. In other words the angle between 2 vectors defined by the head direction of the animal and a vector between the position of the animal and the position of the home. We applied the same Rayleigh vector analysis to cells in the Home Direction space, in this case finding no significant cells.

Classification of cells into functional categories

Cells were classified as head-direction cells, pure grid cells, conjunctive grid cells and rest cells, based on their grid score, spatial in-formation and significance of head directionality according to the following criteria:
-Head direction cells: Rayleigh Head Direction vector length > 0.35 & significant head-direction tuning
- Pure Grid cells: Grid score > 0.42 & significant spatial information.
-Conjunctive Grid Cells: pass criteria for both Grid Cells and Head Direction cells.
-All other cells are consider in the Rest category.
Besides the cell classification, for comparison of different sessions for the same cells we used a stability criterion for cell removal if the Z-score of the firing rate for a session goes below
-1.5 of the previous sessions.

Grid cell sliding window correlation analysis

Given the overall global stability of grid cells, in order to compare the population of grid cells in the presence or absence of the home we used a sliding window correlation method as described in Wernle et al (Wernle et al., 2018).
For each cell we start with two normalized rate maps of the cell, one for each condition being compared. We choose a fixed window of size corresponding to the spacing of the grid cell. Starting in one corner of the rate maps, we

correlate between both maps the rate of active bins covered by the same window. We assign this local correlation value to the position of the center bin of such window. By sliding the window along all the bins of the rate maps we end up with a local correlation heat map that allows us to dissect local changes in each grid cell resulting from the introduction of the home. In all cases, non-existent values were removed from the correlations.
To compare the effect of the home in the population we averaged the local correlation heat maps.

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Competing Interests
We declare no competing interests.

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Perspectives for Chapter II

While we did not find 'home-vector-neurons' and no effects of the home location on the head-direction system, we did find intriguing effects of the home cage on grid cells. Upon further investigation we found that the effects are geometrical in nature. Our findings suggest that grids are locally modifiable, not only by the external boundaries of the environment, but by the internal structure. Thus theories (and grid discharge analyses) that conceptualize all grid discharges as an outflow of a rigid hexagonal lattice (Yoon, Lewallen, Kinkhabwala, Tank, & Fiete, 2016) go badly wrong. Especially as other geometrical non-home cage enclosures (cardboard boxes, corridors) led to similar local grid distortions.

Our work suggests that grid cell activity is indeed plastic at a single node level and that their hexagonal quality can be torn apart for individual firing nodes, this falls in line with recent work showing strong effects of external environmental geometry (Krupic, Bauza, Burton, Barry, & O'Keefe, 2015; Krupic, Bauza, Burton, & O'Keefe, 2018). This contrasts directly with the idea of grid cells being a robust hexagonal lattice (Diehl, Hon, Leutgeb, & Leutgeb, 2017) and may have interesting consequences for attractor models of grid cells, since single fields can escape the hexagonality of the grid.

We suggest in the previous chapter that this plastic property of grid cells may be the substrate for vectorial navigation, by actively over-representing important regions of the environment grid cells might be relaying important information to a downstream decoder. It is our hypothesis that the properties of grid cells as we understand them have the capability of encoding for the affordances of space. This hypothesis is difficult to mechanistically prove, however recent developments of artificial intelligence could provide insight into the putative role of the grid cell plasticity we find. By creating artificial agents whose navigation depends on a population of grid like neurons (Banino et al., 2018) one could compare models of grid cell activity that include or lack this plastic feature.

On a less abstract and more biological standpoint our work also gets us closer to understanding how grid cells encode more complex and naturalistic environments. But it is imperative for the neuroscience of spatial memory and navigation to abandon the limitations of small boxes, wires and even lab environments.

We do hope that work like ours will move the spatial navigation field from studying navigation in abstract cue deprived arenas towards more biologically relevant analyses of spatial behavior. It is paramount to probe neural circuits in a context in which the animals need understand the space they are in and use it to their advantage.

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CHAPTER III

Hide and Seek, a novel spatial and social play behavioral paradigm

Overview

As described in the previous Chapter, spatial navigation is engaged during purposeful behaviors. One such behavior is social interaction and play. Juvenile rats are known to play perform rough and tumble play in ways similar as those described in humans. However, little is known about other forms of play and the neural bases of play in general. Interspecies play is a widespread phenomenon with pet owners and has the advantage of allowing some experimental control. In this chapter we use interspecies Hide & Seek to explore the behavioral and neural correlates of a navigational game.

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Behavioral and Neural Correlates of Hide & Seek in Rats

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Abstract

Evolutionary, cognitive and neural underpinnings of mammalian play are not yet fully elucidated. We played hide & seek – an elaborate role-play-game – with rats. Animals did not receive food rewards, but we engaged in playful interactions with them after finding or being found. All rats quickly acquired the game and during seek systematically searched a large 30m^2-room. Rats guided searches by visual cues and memories of past hiding locations. Rats searched for us in small enclosures, i.e. did not consider our size when choosing search locations. Rats preferred non-transparent over transparent enclosures when hiding. Animals were highly vocal at trial beginnings, when finding the experimenter, during playful interactions and when being returned for a new trial, but were silent when hiding. Rats appeared to enjoy the game itself rather than merely the post-finding-play. Rats played by the rules, i.e. their behavior was highly distinct between hide and seek trials. Neuronal recordings revealed intense activity in prefrontal cortex that varied according to trial events (initiation, finding, being-found etc.). Prefrontal cortical activity also varied with trial types (hiding vs. seeking) and might tell animals which role to play. Hide & seek has found little attention in science, but our findings confirm pet-owner-reports that animals enjoy playing hide & seek. Fast acquisition, strategic behavior and game-adequate vocalization patterns– traits, which emerged without specific conditioning – point to elaborate cognitive and neural capacities for hide & seek in rats. We suggest that role-play in general and hide & seek in particular might be very old.

Introduction

Experimental design in neuroscience traditionally demands strict behavioral and stimulus control. This experimental strategy has elucidated motion vision in monkeys (Britten et al. 1992), reward learning (Schultz et al. 1997) and many other brain mechanisms. This type of investigation, which evolves around neural stimulus response contingencies and conditioned behaviors, has provided less insight on play behavior, however.

The reason for this failure might lie in the very nature of play itself. In his defining book on play (2014, originally 1938) the Dutch historian Johan Huizinga defines five characteristics of play, at least two of which (i.e. play is freedom and no profit can be gained from play) are fundamentally incompatible with behavioral control and conditioning.

Alternative frameworks such as Panksepp's idea of affective neuroscience (Panksepp 2004) allowed addressing challenges posed by the study of play. Indeed, a variety of investigators have analyzed play and the study of rough and tumble play has been particularly illuminating (Pellis & Pellis 2007; Pellis et al. 2018). What is intriguing, is the similarity of human and rat rough and tumble play. A key insight has been the finding that rat vocalizations provide rich insight in play behavior. The analysis of ultrasonic vocalizations in context of human-rat playful interactions and tickling has led to the idea that tickling-evoked ultrasonic vocalizations in rats might be an evolutionary precursor of human laughter (Panksepp & Burgdorf 2003). Such advances also enabled us to direct recording and stimulation techniques – which are still only rarely used in play research – to the study of ticklishness (Ishiyama & Brecht 2016). Surprisingly, we found the playful nature of tickle interactions did not obstruct the identification of putative neural correlates of tickle, while the interspecies nature of the interaction allowed for the intervention of an experimenter in the dynamics of play.

In our current work, we study hide & seek, a more complex play behavior. Apart from few exceptions (Trafton et al. 2006) there has been little research on hide & seek, perhaps, because it is a considered to be a children's game. The game is performed in many cultures and typically involves multiple (≥ 3) players. The game usually starts with assigning who's 'it' (i.e. the seeker) and other players hide. Typically, the seeker allows a certain time span for hiding and usually announces the beginning of the search vocally, often with a traditional proverb. Finding of hidden players is also often signaled vocally. After the hidden players are found, a new round of the game is initiated, usually with newly assigned roles and the first player who was found being 'it'.

We studied a simplified 2-player (rat and human) version of hide & seek, in which trial types (hide vs. seek) were blocked. For a number of reasons, playing hide & seek with rats might provide insights in the biological foundations of play: First, humans enjoy hide & seek and have a deep intuitive understanding of the game. Second, web-reports and youtube-videos provided by pet-owners suggest that animals in general and rats in particular enjoy playing hide and seek. Third, the game appears ecologically valid in that both hiding and seeking abilities are relevant to survival. Fourth, hide and seek is a very elaborate yet easily recognizable role-play. Fifth, richness and complexity of hide & seek afford rules. According to Huizinga (2014) such rules (or order in his terminology) are another defining yet poorly understood characteristic of play.

Specifically, we ask the following questions: Can rats play hide & seek? If yes, how do they play it? What are potential neuronal mechanisms of role-play?

We find rats are skillful hide & seek players and play by the rules. Prefrontal cortex neurons are selective for game-events. Prefrontal cortex neurons also signal trial types (hiding vs. seeking) and may thus instruct role-play.

Results

Hide and Seek

We played hide & seek with rats in a large 30 m^2 room. Preceding a seek trial (Figure 1: magenta) the experimenter allows the rat to jump in the start box (Figure 1 A). Closing the lid of the start box (Figure 1B) signaled to the animal that we assigned it as the seeker and prevented visual cues during the experimenters hiding (Figure 1C, D). Seek trials followed a more or less stereotyped sequence of behaviors. First, the experimenter remotely opened the lid of the box. Shortly thereafter, the rat jumped out of the box and searched for the experimenter (Figure 1E). We considered the experimenter to be found once the rat approached the experimenter < 40 cm distance, having a clear line of sight; we refer to this event as finding (Figure 1E). After finding, the experimenter started a playful interaction (Figure 1F) before carrying the rat back to the start box (return) (Figure 1G). For each trial, search time of the rat was limited to 150 s, starting with the opening of the box. If the animal did not find the experimenter within this time, we considered the trial a failure and the animal was carried back to the start box without playful interaction.

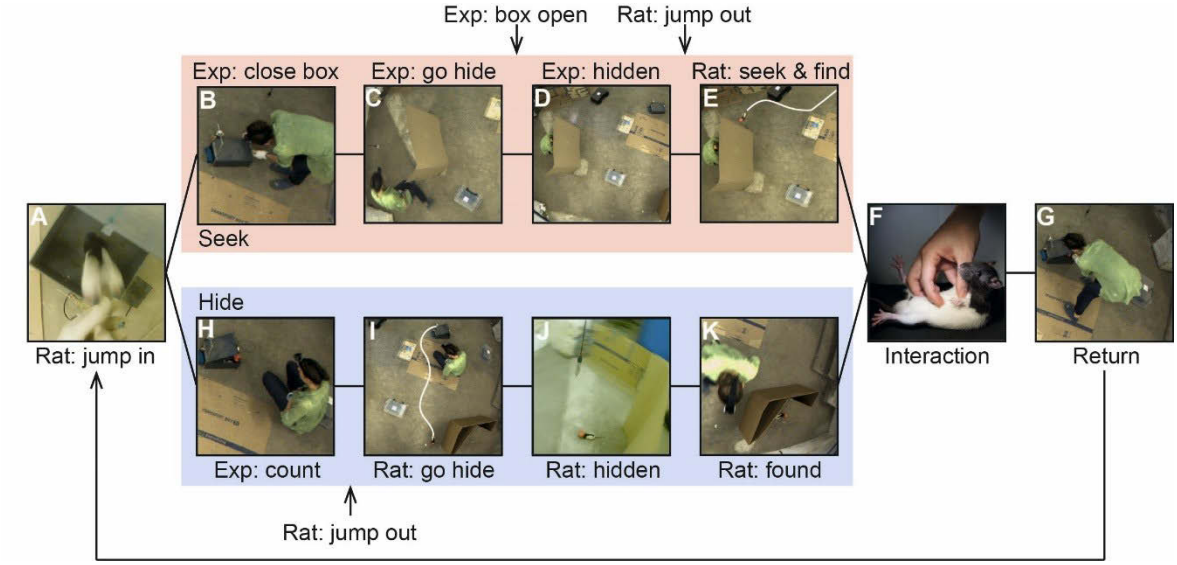

Figure 1, Hide and Seek paradigm. A) The rat jumps into the box to start either a 'Hide' or a 'Seek' trial. For 'Seek' (Magenta) **,B**) The experimenter closes the start box to signal a 'Seek'trial. **C**) Experimenter goes hiding, at arrival the experimenter remotely opens the start box. **D**) While the experiment is hidden the rat proceeds to Jump Out of the box. **E**) The rat seeks and finds the experimenter (Finding). **F**) The experimenter conducts playful interaction with the rat, including tickling, hand chase and other forms of play. **G**) The rat is returned to the start box to start a new trial. A 'Hide' trial (cyan). **H**) The inmobilty of the experimenter by the starting box cues the rat fot the beginning of a 'Hide'trial. **I**) The rat goes to a hiding place. **J**) The rat is hidden from the experimenters view. **K**) The rat is found by the experimenter resulting in an interaction and return for completion of the cycle.

In alternating blocks of trials we reversed roles of the game and assigned the rat as the 'Hider'. Such 'Hide' sessions (Figure 1: cyan) were played in the same room, yet with slightly modified behavioral arrangements. The rat was cued that we assigned it to hide by leaving the start box open and the experimenter sitting immobile next to it (Figure 1H). In the following 90 s, the rat could jump out of the start box, explore the room and chose a hiding location (Figure 1I, J). We offered seven hiding locations, but would also accept if rats moved to hidden areas in the room. Upon successful hiding, the experimenter sought, found the rat (Figure 1K) and initiated the playful interaction, analogous to 'Seek' (Figure 1F). At the end of the trial, the rat was returned (Figure 1G) back to the start box to initiate the next trial.

Rat Seeking Behavior

We tracked the rat's movements during 'Seek', classified the rat's behaviors and show this classification color-coded on the animal's trajectory (Figure 2 A). During the search, animals showed slower, meandering movements and sniffing behavior (which we refer to as exploration) and directed, fast spurts on a straight trajectory (which we refer to as darting).

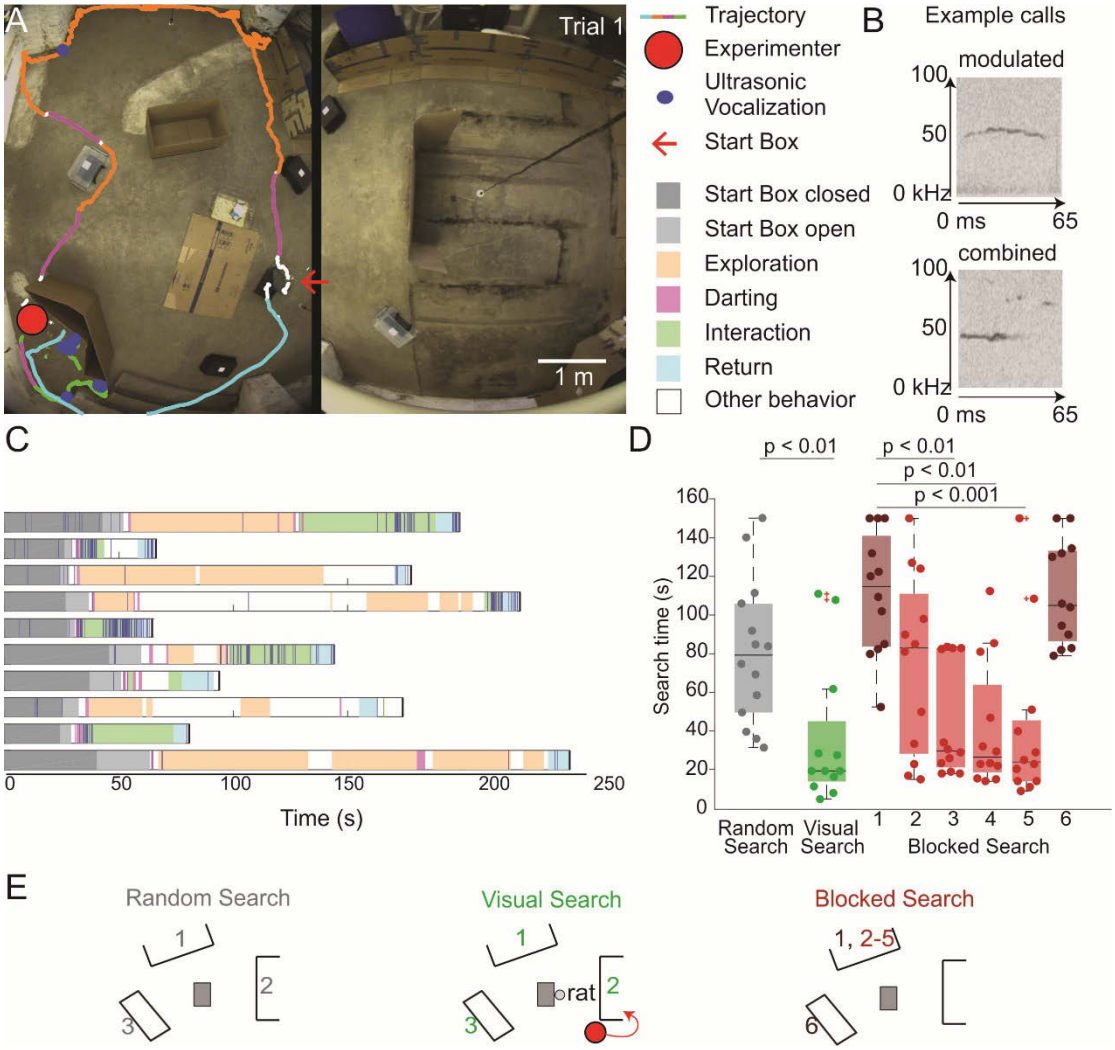

Figure 2: Seeking Behavior

A, Behavioral setup and trajectory of a seek trial. The rat is initially placed into a start box (center), while the experimenter is hiding. Once the experimenter opens the box remotely the rat searches the room (trajectory) until it finds the experimenter and is rewarded with a playful interaction. At the end of the round, the rat is carried back to the start box. Note the clustering of the animal's ultrasonic vocalizations (USV, blue dots) at the beginning and end of the round and when the experimenter is found.

B, Spectrogram of two example calls. One modulated and one combined call are shown.

C, Example seek session, superimposed with ultrasonic vocalization (blue lines). One seek session consists of several trials composed of the following phases: start box closed (rat in the box, lid closed), start box open (rat in the box, lid open), exploration, darting, in case of finding play interaction, return to start box. Other behavior includes resting and sniffing.

D, Median search times over several sessions for 'Random Search', 'Blocked Search' and 'Visual Search' across all animals (data refer to 12-14 sessions per protocol from 6 animals; statistical tests: Wilcoxon rank sum test, Quade test for 'Blocked Search').

E, Different types of seek trial protocols. Left, 'Random Search'. Middle, 'Blocked Search', where the experimenter hides five times at the same location and then changes to a new place. Right, 'Visual Search', where the start box is not closed and the animal can see the experimenter hiding. Each session includes 10-18 trials.

We also recorded the animal's ultrasonic vocalizations and marked the occurrence of ultrasonic calls as blue dots on the trajectory. Spectrograms of such ultrasonic vocalizations are shown in Figure 2B. All animals emitted a variety of ultrasonic vocalizations in the 50 kHz range including flat, modulated and combined calls, as well as ramp up, ramp down, short and bow calls. Animals never emitted 20-30 kHz fear calls while playing 'Seek'. The presence of 50 kHz calls and the absence of fear calls indicates a positive emotional state of the animals during the game (Panksepp & Burgdorf 2003). We provide a schematic representation of behaviors (color-coded as above) and ultrasonic vocalizations (blue dashes) in a series of seek trials in Figure 2C. The duration of the single phases varied from trial to trial (Figure. 2C). The animal spent most time exploring the room, presumably seeking the experimenter.

In order to understand the cues that guided the rat's seek behavior we devised several different seek protocols (Figure 2D). Among these, 'Random Search' is closest to the way humans play hide & seek, with the experimenter randomly hiding behind different hiding places. In the 'Visual Search' protocol, we left the box open and hid visibly for the animal. In 'Blocked Search', the experimenter hid five times in a row behind the same hiding place and then changed to a new location.

Search times (measured as the time between box opening and finding) differed between these seek protocols (Figure 2E). Specifically, we observed significantly longer searches in 'Random Search' than in 'Visual Search', indicating that rats use visual cues in seeking. In 'Blocked Search', we observed a striking drop of search times with hiding location repeats and a significant increase associated with change of the hiding location (Quade test; $p < 0.001$, $n_{session} = 12$, $n_{trial} = 176$). This observation suggests that rats also use memories of prior hiding locations to guide their search.

The analysis of failures rates in different seek protocols led to similar conclusions (Supplmentary Figure 1). Failures were reduced by visual cues and memories of prior hiding locations.

Rat hiding behavior

We conducted tracking, behavioral analysis and ultrasound recordings during 'Hide' trials in the same room (Figure 3A). In Figure 3B we schematically show ten trials of 'Hide' which belong to the same session as the 'Seek' trials shown in Figure 2. The rat spent less time exploring the room and instead spent more time hidden from the experimenter.

Comparing search times with times to hide, rats generally were hiding faster than seeking (Wilcoxon rank sum test; $p < 0.0001$; hide: $n_{session} = 15$, $n_{trial} = 157$; random search: $n_{session} = 14$, $n_{trial} = 182$). The median time to hide over all sessions was 19 s (± 8.25 s).

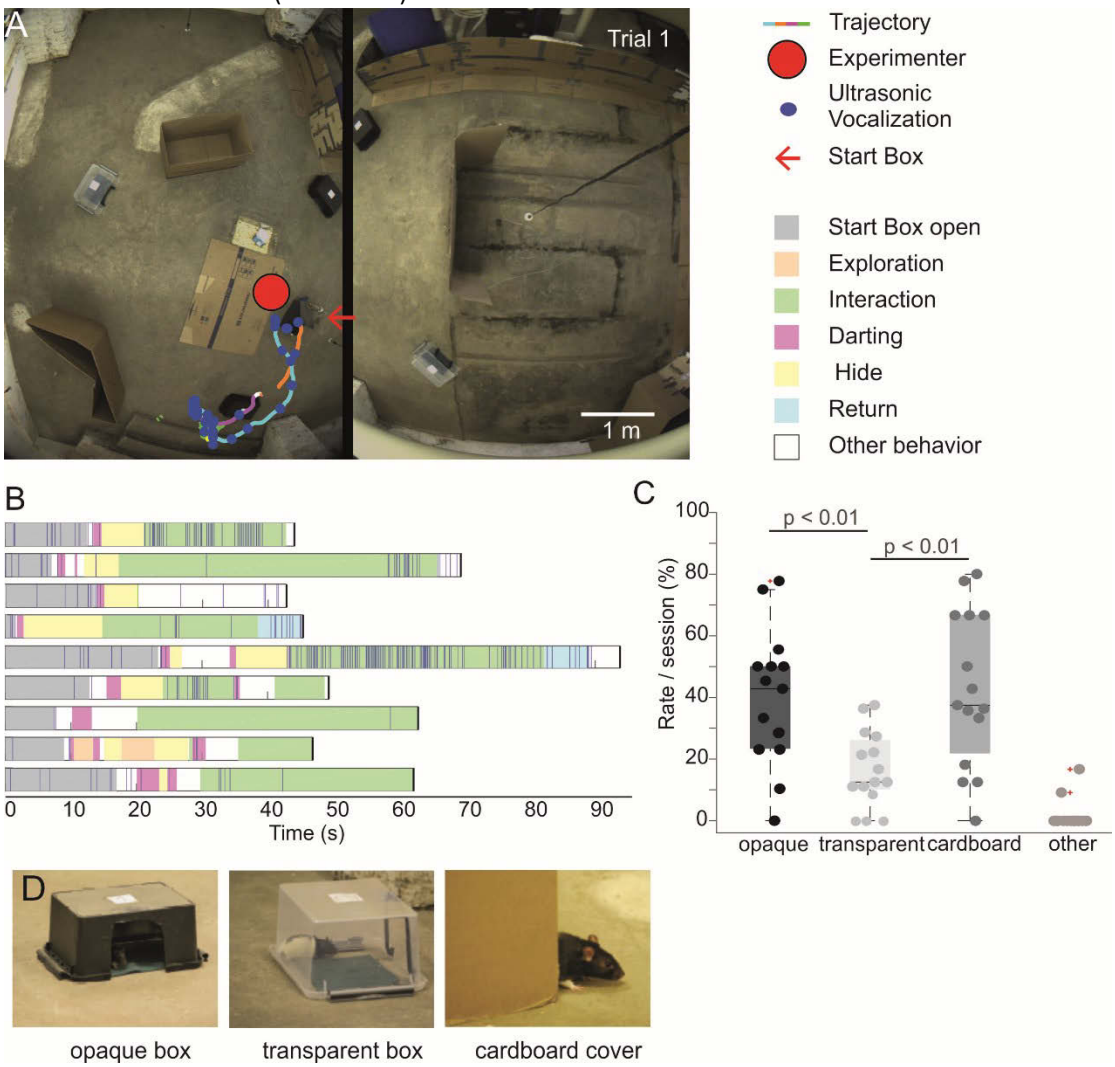

Figure 3: Hiding Behavior

A, Behavioral setup and trajectory of a hide trial. The rat is initially placed into the start box (center), while the experimenter sits next to it. The animal has 90 s to jump out of the box and search a hiding location (trajectory). Once it is hidden, the experimenter is coming to the hiding location and starts a playful interaction. At the end of the trial, the rat is carried back to the Start Box. Note the clustering of the animal's vocalization (blue dots) at the beginning of the trial and during interaction.

B, One example hide session, superimposed with ultrasonic vocalization (blue lines). One hide session comprises several trials that involve the following phases: Start Box open (rat in the box, lid open), exploration, darting, hide (rat stays in a hiding location), after successful hiding playful interaction, return to start box.

C, Usage of different types of hiding locations by the rats (Wilcoxon rank sum, bonferroni-corrected; $n_{session} = 15$, $n_{trial} = 157$, $n_{animal} = 5$).

D, Top: different types of hiding locations: the rat was offered 2 transparent boxes, 2 opaque boxes and the large hiding places also used for hiding by the experimenter.

Similarly, failures rates per session during 'Hide' were generally low with only 10 percent failures. Failures are defined as either the rat staying for > 10 s at a location clearly visible to the experimenter or as other failures. Such other failures included the rat leaving the hiding place before the experimenter arrived, not hiding within 90 s or staying directly with the experimenter for > 30 s.
We further characterized rats' hiding behavior by investigating preferences for different hiding locations. Out of the three different types of hiding locations offered (2 dark boxes, 2 transparent boxes, 3 cardboard hiding places; Figure 3C), rats showed a significant preference for opaque boxes and cardboard boxes over transparent boxes (Figure 3D). Because opaque boxes differed from transparent boxes only in visibility this observation may indicate that rats consider visibility in choosing hiding locations.

Playing and learning hide & seek

Comparing the rat's strategies to seek and to hide, we looked into tendencies to first go to past hiding places (of either the experimenter in 'Seek' or the rat in 'Hide'). When hiding, rats use their last hiding location less frequently (median: 22 ± 11 %) than during their search for the experimenter in 'Random Search' (median: 45 ± 15; Wilcoxon rank sum test; $p < 0.001$).

Out of six male rats that were taught to play Seek, all were able to learn the behavioral paradigm within one to two weeks. Similarly, five out of five rats were able to learn Hide within one week. All rats that learned 'Hide' were then successfully trained to switch between 'Hide' and 'Seek' within one session.

Comparing vocal behavior during 'Hide' and 'Seek'

We studied the expression of different vocal behaviors related to specific events that occur in both, 'Hide' and 'Seek' (Figure 4, seek in magenta, hide in cyan). Given the ultrasonic nature of these vocalizations, the rat's behavioral preferences were not readily accessible to the experimenter while playing the game.
Figure 4A and 4B show the vocal behavior of a subject rat in the same single example session. During 'Seek' rats vocalized in relation to important events in the game, such as jumping into the box, jumping out of the box, finding the experimenter or after interaction, as is event from the example call rasters and peristimulus time histograms (Figure 4A). While call rates are very similar

'Hide' for some events like jumping into the start box, there are also events that seem to elicit different vocalization behavior in the two trial types (Figure 4B). Interestingly, even though vocalization rates increase for 'Hide' and 'Seek' at the beginning of interaction, there is a vocalization peak during 'Seek' preceding the start of interaction (Figure 4A, 4th panel), that does not appear in 'Hide'(Figure 4B 4th panel). To provide further details on this observation, we investigated vocalization behavior during sighting of the experimenter in both trial types, corresponding to the critical moments of the game of 'Hide & Seek' of being found (Figure 4B 3rd panel), and finding respectively (Figure 4A 3rd panel. Interestingly, the peak observed during 'Seek' corresponds to an increased call rate during sighting/finding. In contrast, call rates during 'Hide' drop when sighting the experimenter or being found. This difference was also observed in the average vocalization peristimulus time histograms for all sessions (Figure 4C 3rd and 4th panel).

Further differences in vocalization behavior during 'Seek' and 'Hide' can be found when the animal jumps out of the start box and at the start of darting, with higher call rates during 'Hide' (Figure 4C).

Vocalization rates also varied between different phases of the game (Figure D). In 'Seek', vocalization rates during interaction are significantly higher than during exploration, while the animal is presumably searching for the experimenter (Wilcoxon rank sum test, bonferroni-corrected; $p < 0.05$, $n_{session} = 6$). Animals also called frequently during return back to the start box, and during darting. Vocalization behavior during 'Hide' shows similar tendencies with high call rates during interaction and return and significantly lower vocalization rates during exploration. During hiding, rats vocalize only rarely.

In general, call rates differed between animals and varied from less than 300 calls/session to over 900 calls/session.

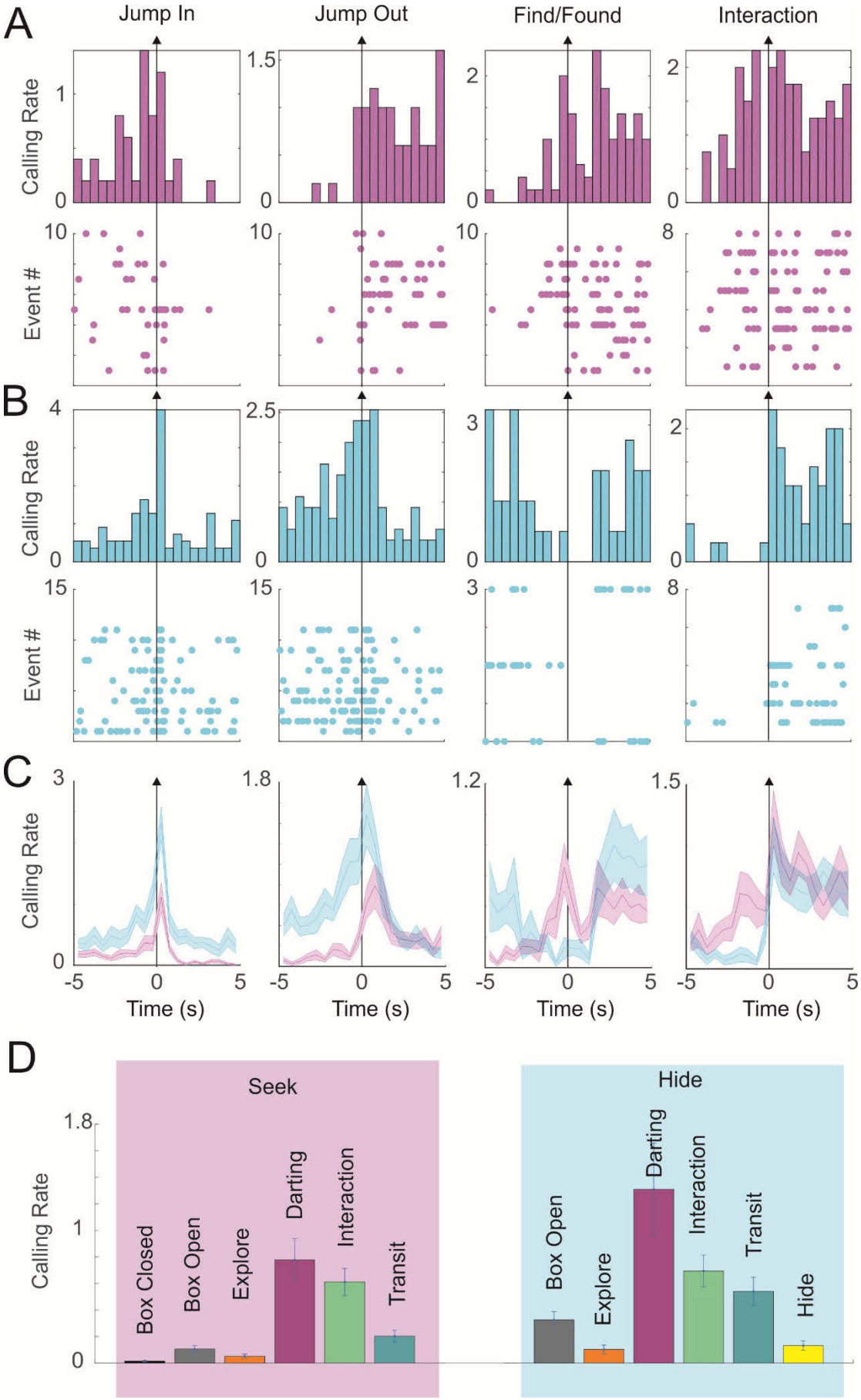
A
Jump In
Jump Out
Find/Found
Interaction
Calling Rate
Event #
B
C
Time (s)
D
Seek
Hide
Box Closed
Box Open
Explore
Darting
Interaction
Transit
Hide

Figure 4: Comparison of Hide & Seek vocalization behavior

A, Rats vocalize at different moments during 'Seek'. Example session of a rat vocalizing at different points in the 'Seek' game. Top row shows peristimulus time histograms corresponding to call rates at the onset of four different game related events, Jump In, Jump Out, Finding the experimenter, and interaction with the experimenter. Bottom row show the corresponding raster plot for timing of each vocalization relative to the event. Note specially that rats start vocalizing while finding the experimenter, before onset of contact.
B, Rats vocalize at different moments during 'Hide'. Example session of a rat vocalizing at different points in the 'Hide' game. Same as above but for Hide. Notice specially that rats don't vocalizing while being found by experimenter.
C, Average vocalization are different between Hide and Seek. Comparison of average call rates for 'Hide' (cyan) and 'Seek' (magenta) at different events of the game (Seek: $n_{session}$ = 14; Hide: $n_{session}$ = 14, n=4). Differences in call behavior are observed for the beginning of interaction, while jumping in/out of the start box, during sighting. Call behavior after interaction onset was similar for 'Hide' and 'Seek'.
D, Calling Rate for different phases of the game. Calling rate for different behavioral phases in all sessions (Seek: $n_{session}$ = 14; Hide: $n_{session}$ = 14, n=4). Darting, transit and Interaction have the highest rates, while exploration and hiding had low vocalization rates. For the Seek session. Rate during explore is significantly lower than: Darting ($p<0.001$), Interaction ($p<0.001$),Transit during Return ($p=0.0141$). For the Hide Session: Rate during explore is significantly lower than: Darting ($p=0.04$), Interaction ($p<0.001$),Transit during Return ($p=0.047$). Rate during Hide is significantly lower than: Darting ($p=0.027$), Interaction ($p<0.01$),Transit during Return ($p<0.01$). Wilcoxon ranksum (pairwise) with manual bonferroni correction.

Neuronal correlates of hide & seek

We investigated the neural correlates of hide & seek by performing wireless tetrode recordings of single neurons (n=177) of the medial prefrontal cortex of rats, a brain area previously associated to encoding of both social proximity (Lee et al., 2016) and encoding of task rules (Karlsson et al., 2012). We looked for whether neurons in the mPFC show changes in their activity according to different phases of hide & seek. Firstly, we found numerous neurons varying their activity sharply during phases of the game (Figure 5A and B, example traces). Cells were preferentially active during different phases of the game including the "Closed Box", "Open Box", Darting, Interaction and Return phases. Some cells were selectively active for a single phase of the game (Figure 5A), while others were of mixed selectivity and responded to multiple events (Figure 5B). The example shown in Figure 5A (1*), corresponds to a cell exclusively responding to closing the start box (Figure 5C), an event that only occurs in seek trials (Figure 5A *1, magenta shaded) . There was a strikingly large respond of the cell to that event, causing an almost 15-fold increase in firing rate (Figure 5C) that only subsided once the starting box was opened (Supplementary video). Cells with a similar response patterns were observed

across animals (Figure 5A *2), including off responses, and transient responses.
Figure 5D shows peristimulus time histograms of the spiking activity of a mixed selective cell (from Figure 5B *3), which shows clear changes in firing rate to multiple events in the game including when the rat jumps into the start box, when it jumps out, when the rat finds the experimenter and when the rats is returned to the start.

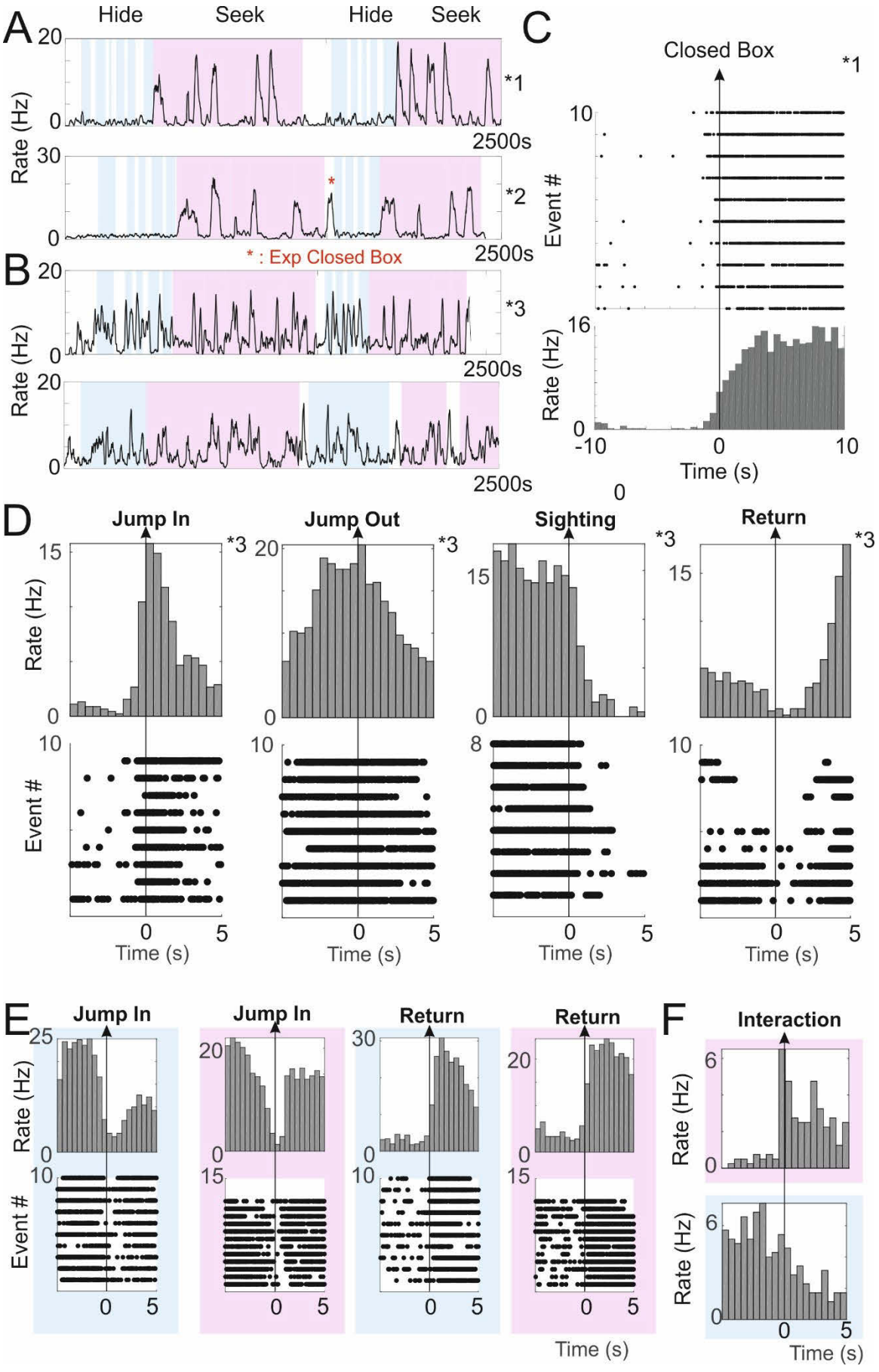
A
Hide
Seek
Hide
Seek
Rate (Hz)
*1
*2
2500s
* : Exp Closed Box
B
*3
C
Closed Box
Event #
Time (s)
D
Jump In
Jump Out
Sighting
Return
E
Jump In
Jump In
Return
Return
F
Interaction

Figure 5: Neuronal correlates of Hide & Seek
A, Firing rate trace of two example neurons showing highly selective responses throughout a complete hide & seek session, divided by different trial types. Note the peaks of high activity specific to 'Seek' trials.
B, Firing rate trace of two example neurons showing mixed-selective responses throughout a complete hide & seek session, divided by different trial types. Note the peaks of high activity are more numerous and non-selective.
C, Activity of selective cell. Raster plot and peristimulus time histogram of all 'Seek' trials. Firing rate of the example neuron (*1) is locked to closing the start box.
D, Activity of mixed-selective cell. Raster plot and peristimulus time histogram of all 'Seek' trials for a mixed-selective neuron (*2). The example neuron has sharp changes in activity correlating to the events of Jump In, Jump Out, Sighting the experimenter and beginning of return transit.
E, Example cell with similar Hiding and Seeking activity. Raster plot and peristimulus time histogram trials for a mixed-selective neuron responding to Jump In and Return/Transit onset. Different color backgrounds demarcate the same cell responding equally to the events in Hide (blue) or Seek (pink) trials.
F, Example cell with differential Hiding and Seeking activity. Peristimulus time histogram trials for a neuron responding to onset of interaction differently in Hide (blue) or Seek (pink) trials. Figure 6

We compared event related activity of cells to events occurring in both 'hide' and 'seek'. Figure 5E shows a cell responding equally to the same events of Jumping into the box, and at the onset of return, for both 'hide' and 'seek' trials. Some cells however, showed context dependent responses (Figure 5F) In order to have a better look at the activity of the population we time warped the activity of each cell according to the median length of a single trial, at the same time conserving the alignment of 4 events, jumping out of the box, onset of interaction, onset of return and jump into the box. This allows us to quantify the average firing rate of cells across trials, as well as compare the activity of all the recorded neurons under the same time scale. Each row of Figure 6A shows the firing rate of a single neuron (n=177) during seek trials normalized to its maximum rate. The neurons are ordered according to the optimal order of a hierarchical clustering (left: classification tree) looking at pairwise correlations between different cells. It is evident from Figure 6A that most of the population activity cares about the phases of the game (demarcated with colored lines), and that different groups of cells are actively engaged in different phases. Including cells being activated by jumping out, onset of interaction and return. To view this further, we clustered the neurons using their 'seek' activity into five groups (Figure 6A hot color code of the clustering tree). The average activity during seek' of these five 'Seeking Clusters' can be seen in Figure 6B. We found clusters are related to different phases of the game. For example, the red cluster seems to be composed by neurons active when the animal is in the box, and includes both example cells in Figure 5A. The yellow cluster is predominantly active when the rat is returned to the starting box.

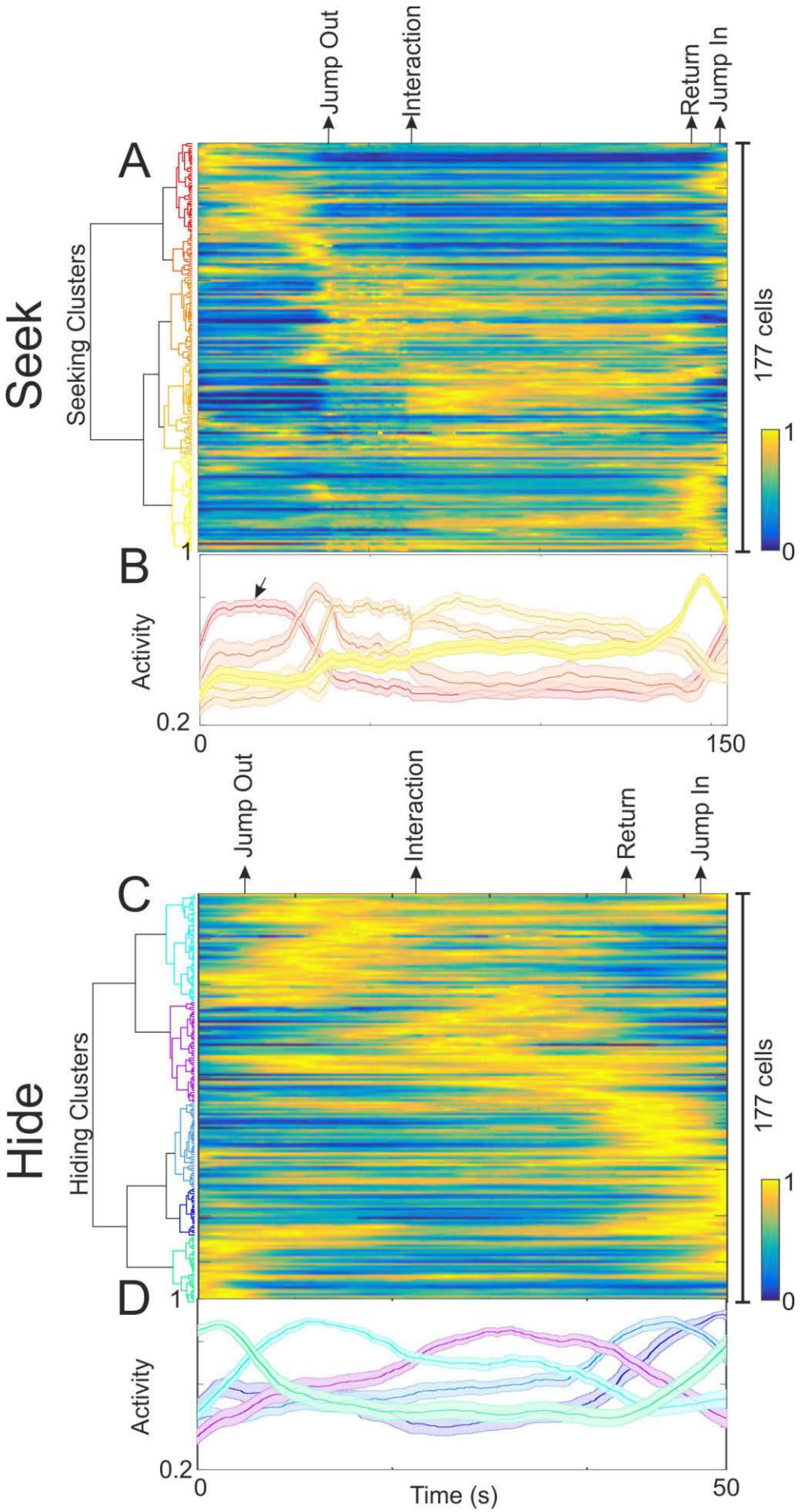
Seek
Jump Out
Interaction
Return
Jump In
A
Seeking Clusters
177 cells
1
0
1
B
Activity
0.2
0
150
Hide
Jump Out
Interaction
Return
Jump In
C
Hiding Clusters
177 cells
1
0
1
D
Activity
0.2
0
50
Time (s)

Figure 6: Population activity during Hide & Seek

A, Population of mPFC neurons are selectively active in different phases of the Seek game. Panel shows time aligned normalized average firing rates for (n= 177) neurons recorded in mPFC. Each row corresponds to a single neuron, and they are sorted according to the results of a hierarchical clustering (dendrogram left) applied to the Seek sessions. Arrows demarcate the occurrence of behavioral events of Jumping Out, onset of interaction , onset of return and Jumping In to the starting box . Box cells from figure 1 are demarcated with asterisks

B, Average firing rate of clusters show different population responses during the Seek game. Mean firing rate and SEM over time during Hide of clusters of neurons selected in A (hot colors corresponding to those of the dendrogram in A). Notice the activity of cluster 1 (red) related to the presence of the animal in the box with the box closed (pointed with arrow). Notice the activity of cluster 5 (yellow) temporally related to the return/transit of the rat to the box. Other clusters show increases related to onset of interaction and jumping out of the box.

C, Population of mPFC neurons are selectively active in different phases of the Hide game. Panel shows time aligned normalized average firing rates for (n= 177) neurons recorded in mPFC. Each row corresponds to a single neuron, and they are sorted according to the results of a hierarchical clustering (dendrogram left) applied to the Hide sessions. Solid vertical lines demarcate the occurrence of behavioral events.

D, Average firing rate of clusters show different population responses during the Hide game. Mean firing rate and SEM over time during Hide of clusters of neurons selected in A (cold colors corresponding to those of the dendrogram in C). Notice the activity of cluster 1 with high activity in the hiding phase of the game.

We next performed the same analysis, to the same neurons, but taking into consideration only their activity during 'hide' (Figure 6C). The activity of the same neurons ordered now according to their activity during 'Hide', also form clear 'Hiding Clusters' preferentially active in relation to different phases of the game. Notice that hiding trials happen in a third of the time, and the activity is aligned to the median time of occurrence of events during 'Hide', which means some of the phases will appear more blown up temporally than in Figure 6A. Figure 6D shows the average activity during 'Hide' of five 'Hiding Clusters' also presenting activity related to different phases of the game.

We were interested in whether mPFC encodes both games in a similar way, or whether there is some information about the differential role the animal plays in the game. In order to explore this we looking at the activity during 'Seek' (Supplementary Figure 1E) and 'Hide' (Supplementary Figure 1F) ordered by clustering the activity of neurons in the opposite role of the game. Doing so did not fully recover the ordered activity seen in Figure 6A. This might imply, that as seen in Figure 5E, some cells are encoding the events irrespective of the contextual role of the rat, and irrespective of the very different temporal demands of the task. However, some cells are differentially active for the same events when the rat plays 'hide' or 'seek'.

Discussion

Summary

We played hide & seek with rats and found them to be gifted and strategic players that systematically searched for us during seek and effectively used covers during hide. All animals showed elaborate vocalizations and played in the absence of food rewards. Neuronal recordings in prefrontal cortex show that this high–level brain structure is heavily engaged in play and that prefrontal activity might instruct role-play.

Rats play Hide & Seek

A synopsis of our behavioral observations strongly suggests that rats indeed played hide & seek. Both, seek- and hide-strategies of the animals make intuitive sense. With respect to seeking such strategies included (i) the systematic exploration of the arena, (ii) the use of visual cues and (iii) memories of past hiding locations. Hiding strategies included (i) the preference of non-transparent enclosures over transparent ones and (ii) opposite to animals' seek behavior, rats avoided their last hiding location.

It also appeared that rats by large played by the rules, another hallmark of play behavior (Huizinga 2014). More specifically, they engaged in entirely different behaviors, when assigned seeker or hider. Such adherence to rules is essential to play and it is non-trivial rats behaved so well. In particular is important to understand that – by the design of our hide & seek paradigm – the human player had only very limited means to enforce such adherence to the rules, because the human was hiding, when rats were supposed to seek, and was immobile, when rats were supposed to hide.
Another hallmark of play is that neither seeking the human experimenter, nor hiding from their behavior fulfilled a function apart from being part of the game, i.e. playing hide & seek is largely pointless. As discussed below also the animals' vocalization behavior and motivational aspects are in line with the play-interpretation of the rats' behavior.

Vocalization behaviour

Rats emitted extremely rich and complex vocalizations during hide & seek and we are only beginning to assess this behavior. Thus, they emitted a wide variety of 50 kHz calls at the beginning of the trial, when finding the experimenter during seek, during the playful interaction and when being returned to the start box. On the other hand, they called very little, when exploring, when hiding and when being found. While we certainly expected animals to be vocal during playful interactions, we were surprised by the vocalizations at the beginning of the trial and during return. From our own subjective recollections of childhood hide & seek, there seem to be similarities between rat and human hide & seek vocalization patterns. Thus, in human hide & seek the seeker often vocally announces the search and often calls out hidden players, when finding them. Moreover, hiding player are also very silent in human Hide & Seek. We stress, however, that more quantitative work is needed on vocalizations in human and rat hide & seek. Further insights into rat vocalization behavior can be gained by classifying different call types. A recent study by Burke et al. (Burke et al. 2017) points towards the importance of ultrasonic calls to coordinate social interactions in rats. Reciprocal calls could be one mechanism by which juvenile and adult rats maintain play.

Why do rats play hide & seek?

Rats appear to enjoy playing hide and seek. Animals engaged in the game, even though no food reward or other conditioning was used. High vocalization rates in the 50 kHz range indicate a positive emotional state of the animals and suggest a low stress level (Wöhr & Schwarting 2013). Several findings suggest that rats are not only enjoying the post-finding play but also the game hide & seek itself. If rats were only seeking and hiding for the reward of playful interaction with the experimenter, they would try to maximize this phase of the game and minimize the length of all other phase, leading to interaction as much as possible. Instead, rats sometimes shortened the interaction phase by leaving the experimenter earlier and re-hiding at another location (video material in supplements), or by finding the experimenter during ‘Seek’ and aborting the beginning of interaction by attempting to tease the experimenter into a chase (video material in supplements). Similarly, rats were occasionally self-initiating ‘Hide’ and ‘Seek’ trials by jumping into the start box (video material in supplements). Further, when animals were given clear cues about the experimenter’s hiding location i.e. by the experimenter not hiding

properly, they often did not take the opportunity to follow the cues for a fast and easy reward but detoured.

An innate preparedness for role-play and hide & seek?

We wonder if our pattern of results is best explained by an innate preparedness for role-play and hide & seek. We don't consider this issue to be settled, but the following observations incline us to think that way. All rats taught to play hide & seek were able to acquire it. Acquisition was remarkably fast (4days reached 70% success), to in case of hiding, rats were capable of playing the game right from the beginning (0 days reach 75% success). Fast acquisition of the game, along with strategic and game-adequate behaviors, many of which we never conditioned the animals to do or of which the experimenter was entirely unaware (such as the game-adequate vocalizations) point in that direction. Based on these findings, as well as similarities with the way humans play hide & seek, we believe that role-play-games in general and hide & seek specifically is an evolutionary old play capacity, which we expect to find in many other species. More generally, we see role playing as being of critical importance to the implementation of other natural behaviors such as mating, hunting and resolution of agonistic encounters.

Hide & seek has found little attention in science, but our findings suggest that this well-played game might be a promising new paradigm to investigate complex social skills and strategic behaviors. Cognitive skills might be assessed in this game, in particular theory of mind related skills. While such skills have been intensely investigated in non-human primates (Gallese & Goldman 1998; Heyes 1998; Seyfarth & Cheney 2013) and children (Lillard 1993; Slaughter 2015), we know very little about such skills in rodents. Only recently, Wiese et al. (2018) could show that activity in the ventromedial prefrontal cortex is related and relevant to mind perception and social attention using human fMRI data. Besides, it is well-known that early prefrontal cortex damage impairs social behaviors in general and social play in specific (Anderson et al. 1999; Schneider & Koch 2005). Moreover, autism spectrum disorder, a disease involving social impairments and altered play behavior, is associated with abnormal prefrontal cortex activity and overgrowth (Naber et al. 2008; Gilbert et al. 2008; Courchesne et al. 2011). More generally, social play as a juvenile is important for further development of social skills (Himmler et al.

2016). Interestingly, it is the prefrontal cortex that seems to mediate an improvement of such skills. Volume of the orbital prefrontal cortex correlates with social cognitive competence, namely intentionality, the ability to predict the behavior of others (Powell et al. 2010).

Medial prefrontal cortex correlates of Hide & Seek

Our tetrode recordings in medial prefrontal cortex revealed large variations in neuronal activity specific to distinct phases and events of hide & seek (i.e. closing of the start box, finding, and interaction). Such correlates of hide & seek in the medial prefrontal cortex support earlier findings that suggest the prefrontal cortex plays an important role in social play behavior (Vanderschuren et al. 1997; Siviy 2016) as well as encoding of rules of the game (Karlsson et al. 2012) (Rougier et al. 2005; Dayan 2007).
Certainly, our results provide only a first glimpse at how play behavior might be controlled. What our results unequivocally show, however, is that under our conditions clear neural correlates of hide & seek can be identified, even though we have typically only few (on the order of 10) trials and the animal behavior is relatively free and hence, temporally variable. The reason for such optimism is the very sharp tuning of medial prefrontal cortex neurons to game events in a similar fashion as described for rats and primates for task events in very short (3-5 s), over-trained and controlled paradigms (Kobak et al. 2016; Karlsson et al. 2012).
Also, the conclusion that medial prefrontal cortex might be involved in play behavior is hardly surprising. In fact, there is almost no behavior, for which an involvement of prefrontal cortex has not been claimed. Still, we find our evidence of trial type (hide vs seek) selectivity in medial prefrontal cortex fascinating. Role play affords very high level control of behavior (i.e. a game-derived idea, what role to play) and medial prefrontal cortex neurons seem to provide such information. Further work will establish if medial prefrontal cortex indeed performs computations essential for role play.

Conclusion

We described behavioural and neural correlates of hide & seek in rats. Hide & seek is a fun and playful behavior. Even though we applied notably less behavioral control than in conventional animal neuroscience (no deprivation,

no food rewards, no fixed trial structure), hide & seek behavior is extraordinary clear and it appears to us that we have an excellent understanding of the animal's thinking in our games. For this reason, we expect the hide & seek paradigm to be a most promising tool in the study of the mammalian mind and brain.

Methods

Animals

Six Long-Evans rats were subjects in this study. We used juvenile male rats, which are known to be very playful (Pellis et al. 1997; Panksepp & Burgdorf 2003). Animals were maintained in a 12:12 h inverted light/dark cycle with free access to food and water ad libitum. Rats were housed individually to increase their social bond to the experimenter as their sole interaction partner (Panksepp & Burgdorf 2003). All Experimental procedures were performed according to German guidelines on animal welfare and local ethics commitees.

Experimental setup

For habituation, training and experiments animals were brought into the 5 m x 6 m illuminated room; animals adapted quickly to the room and the illumination (100 -140 lux). Three hiding places for the experimenter were built using large cardboard boxes and positioned in equal distance to the center of the room. As additional hiding locations for the animals we provided four boxes with an opening made from transparent plastic; two boxes were sprayed opaque. In order to make the boxes more attractive we also included a small piece of cloth. Positions of boxes and large hiding places changed throughout training and experiments. We positioned a lockable 'start box' with a remote cable-controlled opening mechanism sized 32 x 21 x 15 cm at the center of the room (Figure S1). In seek trials animals were locked in the start box, while the experimenter was hiding. To prevent animals from using auditory cues or just follow the remote cable, we included white noise masking while the experimenter went into cover and used decoy cables to all different hiding locations.

Behavioral experiments and habituation

Habituation and training was performed between 10 am and 8 pm during the dark phase of the rats' circadian cycle. Although the overall training strategy was similar for all animals, details of the training protocol were adjusted individually. We either started to train animals on the 'Seek' paradigm or the 'Hide' paradigm. Handling of the animals was done using cotton gloves.

Prior to the training, rats were habituated to the experimenter and the experimental setup starting at postnatal day 21. Each rat was introduced to the experimental environment in its home cage and got offered food reward (peanut butter and fruit loops) during the first 5 days to promote exploration of the room and facilitate habituation. Rats were habituated to the experimenter by 5-10 minutes intensive handling per session. Gradually, the rats' comfort zone increased. Once rats explored the whole experimental environment, the experimenter started with playful interactions, habituating the rat to tickling and hand chasing as described in Ishiyama & Brecht 2016. The whole process took 5-10 days with approximately 1 h habituation per day.

Seek

In the seek paradigm the experimenter places the rat in the start box, locks the box and hides. Then, the experimenter remotely opens the start box and waits for the rat to come and find her. Seeking time for the rat was limited to 150 s. If the rat succeeded in finding the experimenter within the given time frame, it was rewarded by a playful interaction. This interaction involved a play phase of 20-50 s including tickling, hand chasing and rough-and-tumble-like play, as well as petting of the rat. If the rat missed the experimenter, meaning it did not find the experimenter within 150 s, it was picked up and brought back to the start box without playful interaction. A finding event was defined as the rat being in line of sight and less than 40 cm distant from the experimenter. Rats were introduced to seek stepwise. At the beginning, rats were given visual cues. The rat was placed in the open start box, while the experimenter walked away to one of the hiding places. Initially, the experimenter did not hide behind a cardboard box but instead sat down next to it clearly visible to the rat. Once the animals robustly approached the experimenter, the experimenter started to hide. In training, whenever the rat did not find the experimenter within 150 s, the same hiding place was used again in the next round. As a next training step, the experimenter started closing the lid of the start box. Then again, the experimenter would first be visible after box opening before starting to hide fully. Finally, we introduced white noise to mask acoustical cues. We used three different seeking

protocols to characterize seeking behavior. In the first seeking paradigm, 'Random Seek', the experimenter was randomly hiding behind different hiding places. 'Random Seek' is closest to the way humans play hide & seek. To investigate if the rats relied on memories of past hiding locations we devised a second seeking protocol called 'Blocked Seek'. In this protocol, the experimenter was hiding five times in a row at the same location. The sixth time, the experimenter changed to another hiding location. In the third seeking protocol, 'Visual Seek', the experimenter left the start box open and the rat could observe the experimenter while she was hiding. Rats were not conditioned with food or water rewards. Instead, in all seek protocols playful interactions after finding the experimenter were used to teach rats to 'Seek'.

Hide

In the hiding paradigm, rats chose a hiding location and the experimenter sought them. Time to search for a hiding location was limited to 90 s. Much like in the seek paradigm, we played with the rats for 20 - 50 seconds after successful hiding. A hiding trial was scored as successful if the rat went to one of the seven provided hiding locations (2 transparent boxes, 2 opaque boxes, 3 cardboard hiding places) or another hidden area not visible to the experimenter. If the animal failed to hide, it was carried back to the start box without a playful interaction. Two types of failures were distinguished. If the animal went to and stayed longer than 10 s at a place that was clearly visible for the experimenter, this was defined as a visual failure. Other failures included behaviors like the rat going to a proper hiding location but leaving it within 5 s (or before the experimenter found it), the rat not going to any - hidden or visible - hiding location within 90 s or the rat approaching the experimenter and staying within 40 cm reach for > 30 s. After habitation rats were introduced to 'Hide' as follows: the animal was put into the start box, lid open, with the experimenter sitting next to it. As soon as the animal jumped out of the box, the experimenter rewarded it by playful interaction and put it back into the box. This procedure was repeated several times until the animal reliably jumped out. From then on, the animal was not rewarded anymore when it jumped out of the box but only when it went to a hiding location. Initially, the experimenter came to the hiding location very fast (within 5 s). Once the rat did 5 to 10 successful trials, the experimenter took 5 - 15 s to search and find it.

Hide and Seek – task switching

We trained animals sequentially to play 'Seek' as well as 'Hide'. Once the animals mastered both forms of play, we introduced a combined game involving both paradigms. This hide & seek paradigm required the rat to switch between 'Hide' and 'Seek' several times. In these switching sessions, we assigned who seeks and hides and signaled this assignment to the rats by two cues: (1) In seeking trials, the experimenter closed the lid of the start box. (2) In hiding trials, the start box was left open and the experimenter sat immobile next to it, loudly counting. Each game started with 5 rounds of 'Hide', continued with either 5 rounds of 'Random Seek' or 6 rounds (= 1 block) of 'Blocked Seek', proceeded with 5 round of 'Hide' and finished again with either 5 rounds of 'Random Seek' or another block of 'Blocked Seek'.

Video recording and analysis

We acquired wide angle video of the rats' behavior via two overhead Flir Chameleon 3 cameras (FLIR® Systems, Inc., USA) running at 30 frames per second. The images obtained and the corresponding camera metadata related to frame identity and digital input pin states were recorded using Bonsai (Lopes et al. 2015) performing online tracking of the rat.
Videos were manually analyzed for search times, latencies to jump out of the start box, latencies to hide, failures to seek or hide, preferences for different types of hiding locations and probabilities to go to the last hiding location.
Onset and offset times of different behaviors and events where marked using ELAN version 4.9.3 and newer. Specific behaviors were defined as follows:
Darting: Includes all periods where the animal is moving with high speed on a relatively straight trajectory.
Freezing: In contrast to resting, includes times when the animal suddenly stops any movements. In resting, the end of locomotion occurs gradually and the animal may lay down. Both behaviors end with movement.
Exploring: Was defined as periods where the animal moves with low speeds and sniffs the environment.
Jump out: Was defined as the act of leaving the starting box, it starts with moving
over the wall of the start box and ends when all paws touch the ground outside.
Jump in: Was defined when the animal starts moving from the experimenter's hand toward the start box and ends when the animal is inside of the start box.
Grooming: Starts with the first grooming motions on body and ends if no further grooming appears within the next five seconds.

Sighting: Was defined as the time when the animal has a clear line of sight to the experimenter. It ends with either interaction or the line of sight being interrupted.
Interaction: Includes all behaviors, where the experimenter plays with the animal and ends only when the animal leaves the experimenter for at least 20 seconds or return starts.
Return: Describes the time where the animal is carried back to start box by the experimenter and ends with the animal jumping out of the hand.
Hiding: while hiding, the animal is inside an area with presumed intent of being out of line of sight to the experimenter. It ends when the animal comes out, line of sight is established or interaction starts.

Ultrasonic vocalization recording and analysis

Ultrasonic vocalizations (USVs) produced by the rats were recorded using four microphones, one above each hiding place and the fourth above the starting box. For microphones, we used condenser ultrasound CM16/CMPA (frequency range 10-150 kHz) and omnidirectional FG-series electret capsule ultrasound microphones by Knowles (frequency range 10-120 kHz; Knowles Eletronics, LLC., USA). Data were acquired at a sampling rate of 250 kHz and 16-bit resolution using the Avisoft UltraSoundGate 416H and Avisoft-RECORDER software. USVs were visually identified and categorized using Audacity 2.1.2. Categorization followed Ishiyama and Brecht (2016), for detail see supplemental Figure S1 in Ishiyama and Brecht 2016.

Electrophysiological recording and analysis

We implanted 5 Long-Evans rats with Harlan-8 tetrode Drives (Neuralynx Inc., USA) in the medial prefrontal cortex at coordinates (Bregma +3, lateral +0.5). Tetrodes where arranged in a 2 by 4 matrix resulting in recordings between 2.3 mm and 3.8 mm anterior from bregma and 0.25 mm and 0.75 mm lateral from bregma. Tetrodes were turned from 12.5 μm diameter nichrome wire (California Fine Wire Company) and gold plated to 250-300 kΩ impedance. In order to identify tetrodes in the anatomy of mPFC, tetrodes we stained with fluorescent tracers DiI and DiD (ThermoFisher Scientific Inc., USA) before implantation.
We recorded neural signals using a 32 Channel wirefree neural logger developed by Deuteron Technologies recording extracellular signals at 32 kHz. The system consists of a headstage performing amplification and

digitalization, the multiplexed signal is then processed in a processor board and stored on a micro SD card on the head of the animal. The whole system is mechanically attached to the cap of the Harlan-8 drive and covered by a protective case that also served as a red target in our online movement tracking.

The processor board of the Neural Logger receives and transmits radio signals allowing for communication with a base station. Radio communication is fast enough to enable synchronization via TTL's between the base station and the logger. Hardware copies of these TTL's are also sent to the cameras via I/O pins and to the Avisoft hardware to ensure synchronization of all devices.

Extracellular recordings were spike detected and sorted using Kilosort (Pachitariu et al. 2016). After the initial sorting, clusters were manually curated using Phy in Python. Cluster quality was assessed by spike shape, cluster separation of its principal components. SNR and ISI-histogram with lack of contamination in a 2 ms refractory period.

After conclusion of the experiment, rats were anaesthetized with urethane and depth positions on selected tetrodes were marked with electrolytic lesion. The lesions were conducted using a NanoZ (Neuralynx Inc., USA) with a DC current of -8 (μA) for 8 seconds, tip negative. The rats then received an overdose of the anesthetic and were transcardially perfused using a prefix solution followed by PFA at 4%. The brains were extracted and post-fixed in 4 % PFA for 18-24 hours before being sectioned coronally into 100 μm thick sections. Before proceeding with tissue staining, slices were photographed at an epi-fluorescence microscope to reveal differential patterns of fluorescence dyes (DiI or DiO) on different tetrodes. This allowed for further identification of each individual tetrode. To finalize the histology tissues went through cytochrome oxidase staining and were imaged under a bright field microscope to visualize the lesions and identify the anatomical location of each tetrode in the brain in according to the Paxinos & Watson rat brain atlas (Sixth edition, 2007).

Temporal Compression and alignment

For comparing neural activity across cells, we temporally stretched/compressed linearly the firing rate of neurons in segments that would preserve the alignment of four critical game events (Jump Out of the box, Interaction Onset, Return onset, and Jumping Into the box).

All cells where aligned and compress to fit the medians times of the critical events in all cells. This was done as explained in (Kobak et al. 2016).

Hierarchical Clustering Analysis

We used a hierarchical clustering algorithm to order and classify in clusters the neural activity of the population. Pairwise distances between cells where calculated according to their normalized firing rate correlation and the hierarchical tree was built using a ward algorithm. Rows of the matrix were ordered according to an optimal leaf order function. Clustering was restricted to an arbitrary number of 5, for easy comparison and visualization of the data. The analysis was done fully with on board MATLAB subroutines.

Statistical analysis

All data were tested for a normal distribution using the Shapiro-Wilk test and a QQ-plot as a graphical method well-suited for small sample sizes. The Levene-test based on the median of the data was used to test for homoscedasticity and compare variances of two groups. Since most parameters did not follow a normal distribution and were heteroscedastic, the following non-parametric tests were used to investigate differences in median: Wilcoxon Rank Sum test was used to compare two independent groups. A bonferroni correction of p-value was added to compare medians of 3 groups belonging to the same data family. The Quade test was used to test multiple paired groups, using the 'Hommel correction' (Hommel 1989) for post-hoc testing.
Statistical analysis was performed in Matlab (R2018a) and R (Version 3.5.1), using the packages PMCMRplus (1.4.0), moments (0.14), onewaytests (1.8) and car (3.0-2).
Details in text and figure legends are either median ± interquartile range (IQR) or mean ± standard error of mean (SEM) for normally distributed data.

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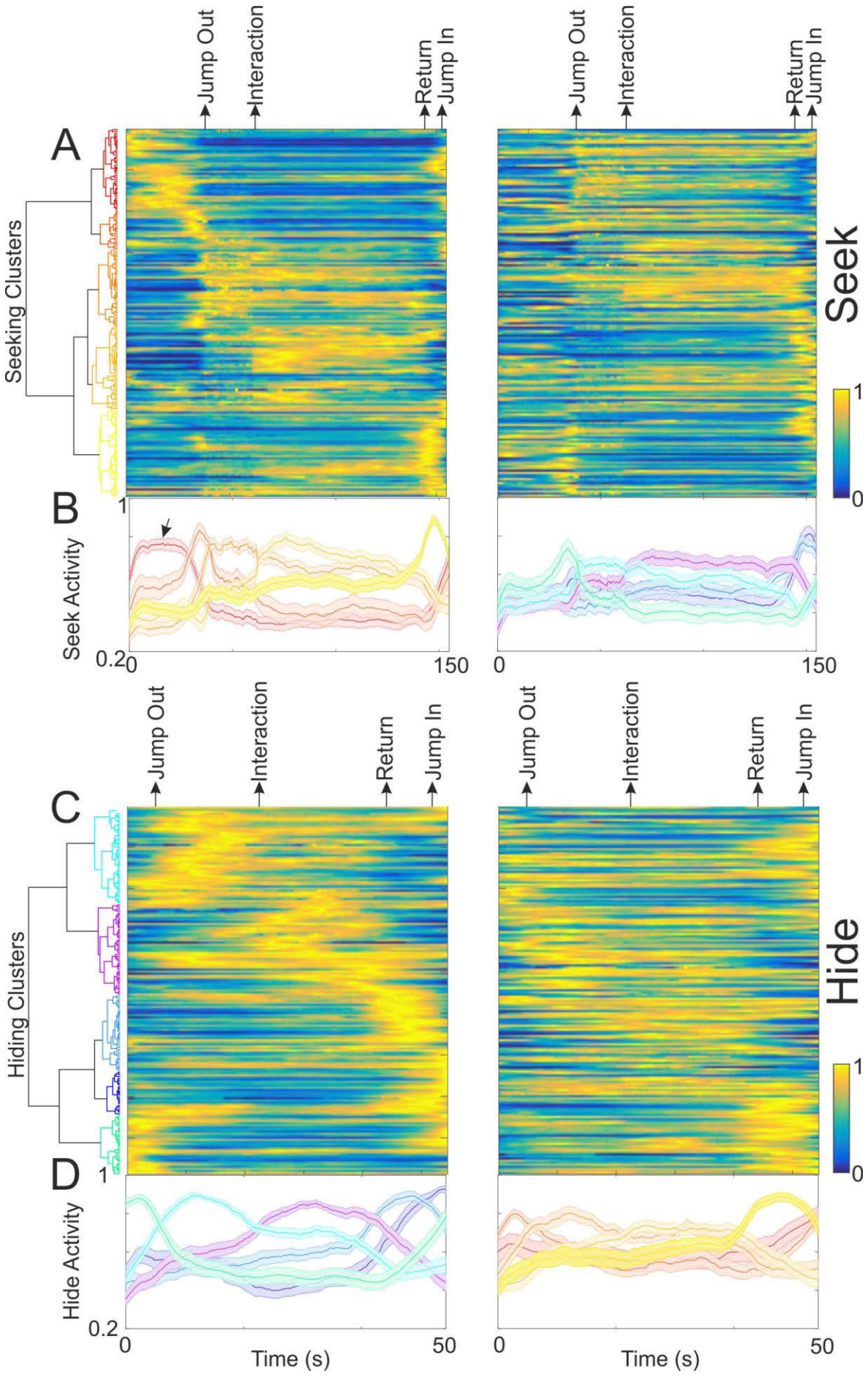

A
Jump Out
Interaction
Return
Jump In
Seeking Clusters
Seek
1
0
B
Seek Activity
1
0.2
0
150
C
Hiding Clusters
Hide
D
Hide Activity
0.2
0
50
Time (s)

Supplementary Figure 1: Population activity during Hide & Seek

A, Population of mPFC neurons are selectively active in different phases of the Seek game. Panel shows time aligned normalized average firing rates for (n= 177) neurons recorded in mPFC. Each row corresponds to a single neuron, and they are sorted according to the results of a hierarchical clustering (dendrogram left) applied to the Seek sessions. Arrows demarcate the occurrence of behavioral events of Jumping Out, onset of interaction , onset of return and Jumping In to the starting box. Box cells from figure 1 are demarcated with asterisks

B, Average firing rate of clusters show different population responses during the Seek game. Mean firing rate and SEM over time during Hide of clusters of neurons selected in A (hot colors corresponding to those of the dendrogram in A). Notice the activity of cluster 1 (red) related to the presence of the animal in the box with the box closed (pointed with arrow). Notice the activity of cluster 5 (yellow) temporally related to the return/transit of the rat to the box. Other clusters show increases related to onset of interaction and jumping out of the box.

C, Population of mPFC neurons are selectively active in different phases of the Hide game. Panel shows time aligned normalized average firing rates for (n= 177) neurons recorded in mPFC. Each row corresponds to a single neuron, and they are sorted according to the results of a hierarchical clustering (dendrogram left) applied to the Hide sessions. Arrows demarcate the occurrence of behavioral events of Jumping Out, onset of interaction , onset of return and Jumping In to the starting box.

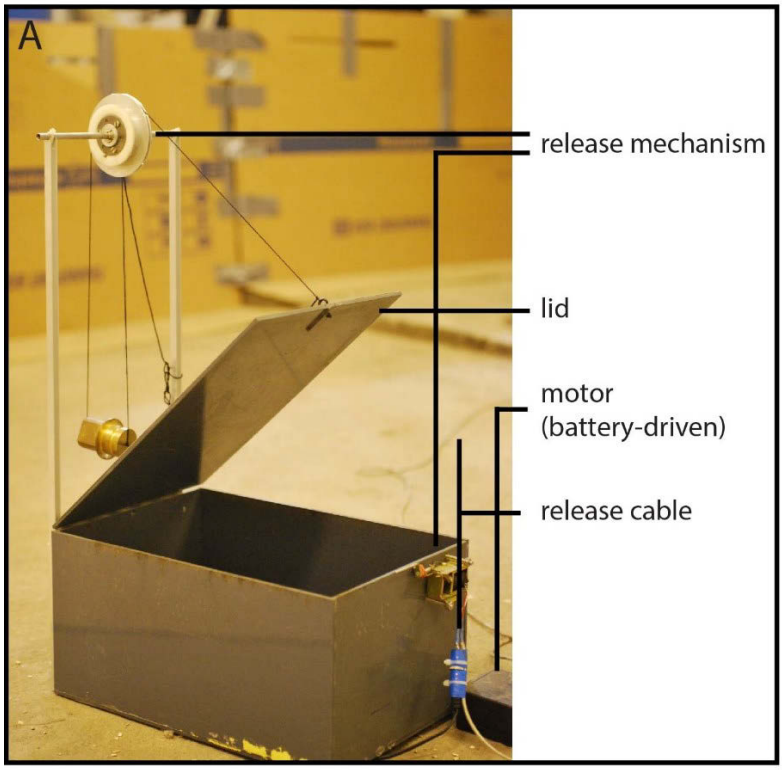
D, Average firing rate of clusters show different population responses during the Hide game. Mean firing rate and SEM over time during Hide of clusters of neurons selected in A (cold colors corresponding to those of the dendrogram in C). Notice the activity of cluster 1 with high activity in the hiding phase of the game.

E, Population of mPFC neurons during Seek sorted according to clustering of hide sessions. Panel shows time aligned normalized average firing rates for (n= 177) neurons recorded in mPFC. Each row corresponds to a single neuron, and they are sorted according to the results of a hierarchical clustering applied to the Hide sessions. Solid vertical lines demarcate the occurrence of behavioral events. The structure is not fully conserved, meaning that the activity of the cells during Hide, can't fully explain the activity of the cells during seek. Only some of the structure is retained.

F, Neurons that cluster during Hide do not show consistent activity during seek. Mean firing rate and SEM over time during Seek of clusters of neurons selected in Hide (cold colors corresponding to those of the dendrogram in B). Notice the activity of all clusters shows less evident differences. Also notice, that the information regarding clusters encoding the closure of the box is lost.

G, Population of mPFC neurons during Hide sorted according to clustering of Seek sessions. Like above each row corresponds to a single neuron, and they are sorted according to the results of a hierarchical clustering applied to the Seek sessions. Solid vertical lines demarcate the occurrence of behavioral events. The structure is not fully conserved, meaning that the activity of the cells during Hide, even though in this case it seems like some clusters for the Seeking sessions do represent the behavior of the neural population during Hide.

F, Neurons that cluster during Seek show some consistent activity during Hide. Mean firing rate and SEM over time during Hide of clusters of neurons selected in Seek (hot colors corresponding to those of the dendrogram in B). Notice the activity of all clusters shows less evident differences than in D. However, some of the pattern is preserved.



Supplementary Figure 2: Start box with remotely controlled release mechanism.

Perspectives Chapter III

The possible ripple effects of this chapter are harder to foresee than the previous two. Firstly, because it truly rests on less solid ground than the previous work. Hide and seek as a behavioral paradigm has never been studied. There is probably little novelty in the behavior. Probably hide and seek as a form of play is very old, and many attentive pet owners could attest to animals, and rat's capacities to play this game. However, scientifically, the novelty of this paradigm is immense. We have shown, not only, that rats are able of playing this sophisticated human role playing game, but also, that they do so with similar strategies and behaviors. Without a doubt, the capacity of rats to play this game speaks to their intelligence and sociality, and sets the stage for using Hide and Seek and play as a way to probe for cognitive skills like theory of mind, decision making, social role play etc.

We have observed that after many hours of experiment and video review, we believe to achieve a good understanding of the behavior and sometimes the intentions of the animal. Our own capacity for theory of mind, which some might call anthropomorphism, allows us to. Some of these observations are related from the rat's behavior based on what it observes. For example, we have seen rats, looking at where the experimenter is searching, seeing the experimenter move on, and deciding to hide where the experimenter has already looked. This is something the animal was not trained to do, it's a one take behavior, a very adaptive behavior.

Of course, such an observation is only that, an observation. But it points toward this free play behavior, as a way to produce adaptive intelligent in rats. Perhaps, hide and seek could be a test bed for studies how deep cognitive homologies exist between rats and humans.

Regarding more specific questions. Hide and seek seems like an idea behavior to further study spatial navigation. We have seen that medial prefrontal cortex is very engaged during different phases of the game, including the navigational part of the game. While coming out of the box, rats make decisions related of places to search or where to hide. We have still not been able to analyze whether mPFC instructs the rest of the spatial circuitry where to go next. We clearly have the navigational decisions made by the rat, and whether those decisions were correct. For blocked sessions, we have data on

the spatial biased accumulated by the rat, on where to search based on past history. Many of these questions are still open and were just put to a side by the clear game related responses found in mPFC, which we believe help the animal navigate the narrative of the game.

In the future, this task is ideal to study the hippocampus, since it engages many of its functions. For starters, the animal is navigating space. Are place cells game dependent? Does a place cell remap when the rat is playing 'Hide' or 'Seek'? The fact that the game is ordered in trials, and has a starting point has interesting repercussions for the study of pre-play and re-play. Is pre-play modulated by the hiding bias of the experimenter?

Multiple questions related to sharp wave ripples and the memory established in the hide and seek paradigm. Sharp wave ripples are a biomarker associated to episodic memory and planning (Buzsáki, 2015). Does the expression of sharp wave ripples between trials or sessions reflect the implementation of different playing strategies?

Few things are as interesting as the role of hippocampus in social memory. Recently social place cells were described in bats and rats (Danjo, Toyoizumi, & Fujisawa, 2018; Omer, Maimon, Las, & Ulanovsky, 2018). Cells that are active for the location of a conspecific in space. Does the rat have social place cells for the position of the experimenter in the room? Are these selective for the identity of the experimenter? Does the activity of these cells help the animal flexibly hide from the experimenter?

Many open questions.

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General Discussion

This thesis is separated into three chapters, which rather than being an artificial order, they reflect a natural progression. Every chapter was conducted once the previous chapter had reached a reasonable conclusion. Each of them has their own discussion, and the perspective that followed. So, now I reserve this modest space in the thesis for a broader discussion and a look into the future.

The three main chapters of this thesis relate to each other temporally. They represent a pattern of exploratory behavior. Akin to the homologous exploration of space across taxa described in the introduction.

It starts with Chapter I using traditional ways to look at the brain, anatomy, circuits physiology somewhat srerile behavioral conditions. Traditional mostly to the lab where this thesis was conducted, which has looked intensely at structure-function relations (Burgalossi & Brecht, 2014; Burgalossi et al., 2011; Ray et al., 2014; Tang et al., 2014, 2015) . This approach allows us to dig deep into the neural circuitry of the brain, to look at single cells, to understand where they are situated in the anatomy of the brain, and how are they connected to other areas around it. The information acquired in Chapter I is of great importance, the level of detail will allow us in the future to intervene the brain's activity in anatomically and histologically specific ways in order to causally test ideas about the role of this circuitries. The use of optogenetics paired with neural recordings will help elucidate the contribution of the parasubiculum to navigation and MEC activity. A contribution that we hypothesized as major, due to the connectivity from PaS to MEC, the internal connectivity of PaS, the precedence in theta-space firing of the PaS in relation to MEC.

Metaphorically of course, Chapter I resembles the exploration of the boundaries of a space done by a mice in a novel environment (Fonio, Benjamini, & Golani, 2009). And returning back to our starting point (trip 8 in

Figure). After having worked in computational neuroethology I entered a novel space, experimental neuroscience, and contributed to a trip around a very interesting brain area.

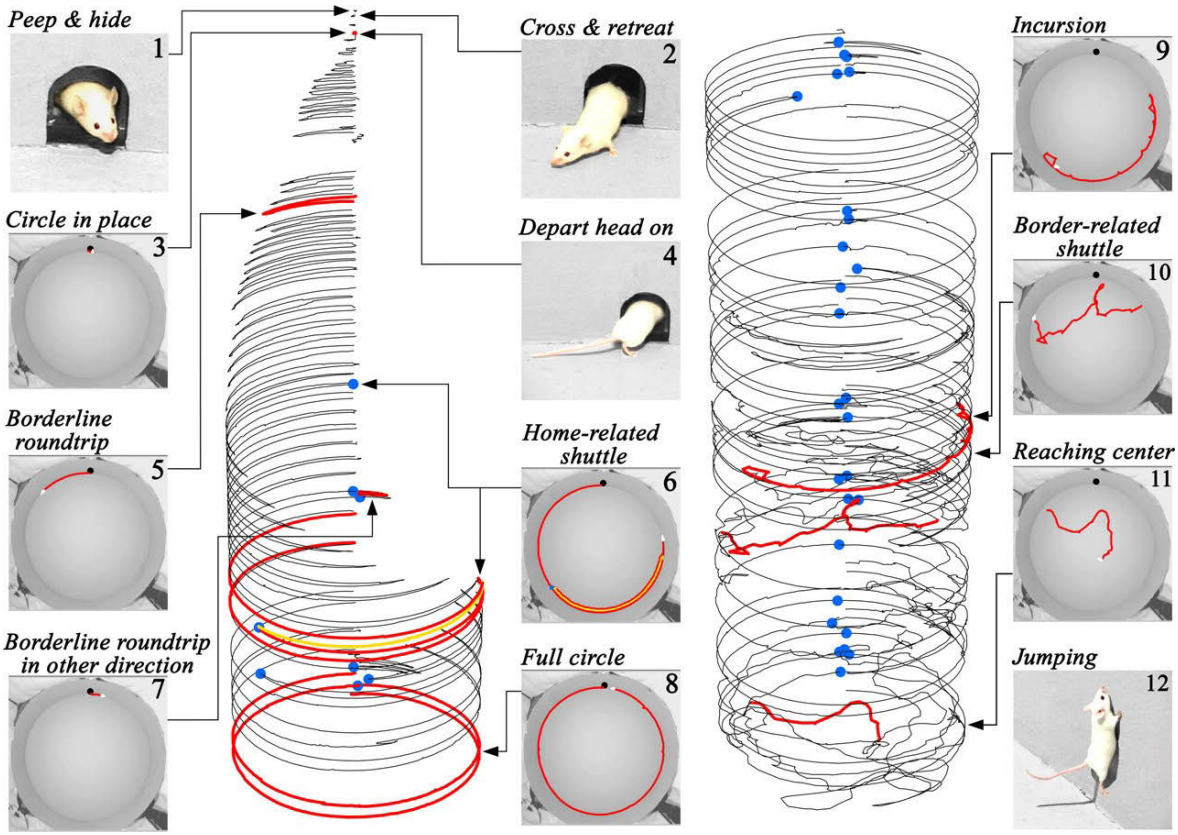

Figure. Sequential trips of a mouse into a novel arena from a initial home location. Notice the existence of behavioral transitions or phases in how the animal explores the dimensionality of the arena.

In Chapter II however, we moved in a direction truly orthogonal to the boundary (like the mouse's incursions to the center of the arena 9, 10 and 11 in the figure), in search for a home vector in the parasubiculum and MEC. Even though we didn't find an explicit representation of home, we encountered, by putting the animal in a more complex context that both head direction cells and grid cells in MEC are very stable to the introduction of the home of the animal in the environment, a result favored by wireless recordings, having the animal be very familiar with its environment, and not attempting to disorient the animal between conditions. Remarkably though, we found that this global stability, of both head direction cells and grid cells, allowed us to probe grid cell plasticity at the level of single fields. The main question that stems from these results is: how do breaks of the hexagonal lattice contribute to the representation of space and the capacity to navigate? Our principal

hypothesis is that grid cells distortions contribute to vector encoding in complex spaces by correlating their activity in geometrically relevant locations. Our results and hypothesis are consistent with hypotheses that grid cells allow for vectorial calculation in the brain (Banino et al., 2018; Bush, Barry, Manson, & Burgess, 2015; Kubie & Fenton, 2009). It is possible, that the hexagonal grid cell phenomenon is a consequence of the brain finding an optimal way to encode all possible vectors in the environment. In an open, homogeneous environment, the result of that optimization are perfect hexagonal grids. However, as has been showed by Derdikman (Derdikman et al., 2009), when a 2D environment has its possible paths restricted in a hairpin maze, the hexagonal grid representation disappears, now, the paths of each hairpin in the maze can be represented with two vectors, back and forth. Clearly, geometry affects the possible paths, and vectorial trajectories one can take in a space. Both the Derdikman paper and Krupic's recent work (Krupic, Bauza, Burton, & O'Keefe, 2018) can be interpreted as changes in grid cells in situations where the possible vectors to be encoded change. However will geometry be the only thing affecting grid cells single fields?

Interestingly, recent work by Boccara and Csicsivari (unpublished, presented at various meetings) shows grid cells moving single firing fields towards goal locations. If these results hold to be true, they show an additional level of grid node flexibility. However, we do think, both things could be related.

Both the internal geometry of a space, and the existence of rewards in certain locations, modify what we interpret as the affordance of space. Stemming from Gibsons'concept of affordances applied to the animals space (J. J. Gibson, 1979; J.J. Gibson, 1966), a concept used for example by architects. Space, like objects, can be used in different ways, and because of its nature, have different affordances. Any kid in Uruguay develops a talent to find places affording a good football match. Not every place allows for such an endeavor. Many things can change the affordance of a space, one such thing is internal structure. It is hard to play football in a hair-pin maze. In a similar way to geometry, the occurrence of reward or resources also change the affordances of space. It changes what paths, vectors, need to be better encoded by the brain.

The conceptual framework of affordances, coined by Gibson in "The senses considered as perceptual systems (1966) and then expanded in "The ecological approach to visual perception" (1979), points us towards studying navigation and space in a context where space is to be used. In his own words:

"The habitat of a given animal contains places. A place is not an object with definite boundaries but a region (Chapter 3). The different places of a habitat may have different affordances. Some are places where food is usually found and others where it is not. There are places of danger, such as the brink of a cliff and the regions where predators lurk. There are places of refuge from predators. Among these is the place where mate and young are, the home, which is usually a partial enclosure. Animals are skilled at what the psychologist calls place-learning. They can find their way to significant places. An important kind of place, made intelligible by the ecological approach to visual perception, is a place that affords concealment, a hiding place."

The ecological approach to visual perception. J.J Gibson, (1979)

Finally, in Chapter III, this thesis explores a final third orthogonal dimension, akin to the vertical dimension in the example shown in the figure related to jumping and climbing the walls of the arena. Driven by the affordances of new wireless technology, and a strong interest in more ethologically relevant and unrestricted behaviors, we embarked upon the study of rats playing hide and seek with humans.

This Chapter of the thesis shows rats are capable of quickly acquiring the game of 'Hide and Seek' from a human, without specific conditioning other than playful interaction. They don't only acquire the game, but they are capable of using different strategies to play the game and they emit without conditioning game appropriate vocalization patterns. This is a remarkable finding that point towards a deep homology in mammalian play behavior. Not a surprise to any pet-owner of multiple species, it is clear that animals of different species are capable of playing together.

The use of different strategies as well implies some level of understanding of the rules of the game, and how they can use the information available to them in order to win. Rats used visual information and memory resulting in finding the experimenter faster. They also hid, more frequently under opaque hiding locations. This again implies, although does not prove, the animal's understanding of the affordances of opaque boxes in the context of hiding. Again, Gibson:

An observer can perceive not only that other observers are unhidden or hidden from him but also that he is hidden or unhidden from other observers. Surely,

babies playing peek-a-boo and children playing hide-and-seek are practicing this kind of apprehension. To hide is to position one's body at a place that is concealed at the points of observation of other observers. A "good" hiding place is one that is concealed at nearly all points of observation.

The ecological approach to visual perception. J.J Gibson, (1979)

Besides these intriguing behavioral findings we have studied the activity of medial prefrontal cortex under these unrestricted, playful circumstances and found clear neural correlates for most game events. We also note that some cells are clearly differentiating their activity between hide and seek. We suggest that prefrontal cortex in the context of hide and seek could be providing information about the role the animal takes in the game. This is a very suggestive idea that requires further experimentation and dissection. But the conceptual necessity for studying role taking is of much interest. Playing in all its forms, locomotive, object play, and social role playing are perhaps ways to explore the affordances of others and of ourselves.

Behavior affords behavior, and the whole subject matter of psychology and of the social sciences can be thought of as an elaboration of this basic fact. Sexual behavior, nurturing behavior, fighting behavior, cooperative behavior, economic behavior, political behavior-—all depend on the perceiving of what another person or other persons afford, or sometimes on the mis-perceiving of it.

The ecological approach to visual perception. J.J Gibson, (1979)

Studying play, with its inherent freedom and spontaneity, is of incredible importance. The stimulus response approach to behavior has clear advantages regarding repetition and control, and has been the basis of important findings about the brain. However, it has serious limitations in explaining perhaps the most interesting behaviors, spontaneity, play, intelligence and creativity. Understanding the 'noise' in behavior (Gomez-Marin, Paton, Kampff, Costa, & Mainen, 2014), the moment the rat does what we didn't expect it to do, like avoid being interacted with at the end of a trial in order to hide again, or jumping into the starting box to start a new trial on its own, requires free paradigms that put the animal in situations where the animal calls the shots, and these behaviors just happen to occur.

In the end it all comes down to behavior, the results of brain activity. And how studying it requires understanding the 'Umwelt' of the animal, and working

as close as possible to the conditions in which the brain evolved to work. This thesis does not necessarily achieve such a goal, but it gets us closer to studying neuroscience of ethologically relevant behavior, and from a personal standpoint sets a bearing for the future.

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Future Perspective

The study of the nervous system is the study of the advantages animals obtain by developing flexible behaviors to interact with their ever-changing environment. However, due to technological limitations, the study of brain has mostly being restricted to the study of tissues or animals in less than ideal settings, whether that is in brain slices, neuron cultures, anesthetized preps, head-fixed experiments, or even lab restricted settings. This is far from ideal, and though it contributes immensely to understanding brain circuitry, it does so in a way that doesn't necessarily fit with the objective of understanding how brain activity explains natural behavior (Krakauer, Ghazanfar, Gomez-Marin, MacIver, & Poeppel, 2017).

Fortunatelly, doing Neuroscience outside of the lab is becoming possible due to the technological advances on several fronts. Recent developments in miniaturization have resulted in fully head born wire-free electrophysiology and optical imaging setups allowing us to monitor brain activity in unrestricted animals (Rattenborg et al., 2016; Sarel, Finkelstein, Las, & Ulanovsky, 2017; Vinepinsky, Donchin, & Segev, 2017).

On the behavioral side, ecologist have been tracking the lives of wild animals for decades. However recent advances in GPS technogy, accelerometers, microphones have taken tracking the behavior of unrestricted wild animals to a new level, even in collectives (Flack, Nagy, Fiedler, Couzin, & Wikelski, 2018; Strandburg-Peshkin, Papageorgiou, Crofoot, & Farine, 2018; Tsoar et al., 2011). Finally, recent developments in the fields of computational neuroscience and machine learning are allowing us to have a better grasp of complex datasets both at the behavioral level (Brown & Bivort, 2018; Egnor & Branson, 2016; Kabra, Robie, Rivera-Alba, Branson, & Branson, 2013; Robie et al., 2017; Todd, Kain, & Bivort, 2017; Wiltschko et al., 2015).

The combination of these developments on miniaturization, logging, freely moving imaging (Ghosh et al., 2011), high density probes for extracellular recordings (Jun et al., 2017), behavioral monitoring and analysis capabilities will, in the short term future, allow us to minimize the ecological impact of

neuroscience experiments in the wild, opening a completely new era in neuroscience.

In this thesis, Chapters II and III have shown the value of using wireless recordings to study truly freely behaving animals. Chapter III sets the stage for a future where we can study the brain of free, behaving, cooperative animals. We have proven that under extremely unrestricted, non classically conditioned behavior, in a sizable environment we were still able to recover the animals neural activity and see the brain perform on a completely different temporal and spatial scale.

This opens up a new avenue for doing Neuroscience outside the lab, and such a technical development opens a panoply of new questions.

I.
How is space represented in the wild? The spatial navigation field has developed tremendously thanks to the development of freely moving extracellular recordings. However, its development seems to be curtailed by the fact that it's not studying real navigation. In general studies on the spatial navigation circuit are conducted in labs, with animals that have never seen the light of day, which are recorded while freely roaming in a cue deprived environment. Even though this approach has resulted in some amazing findings, it has also biased strongly our understanding of how animals navigate. Grid cells, for example, were not long ago thought to be a crystal structure in the brain, now found to be, by the work in Chapter II and other amazing recent work from O'keefe and Krupic (Krupic, Bauza, Burton, & O'Keefe, 2018), a very flexible non stable lattice which adapts to the complexities and changes of environments.
A burning question in this field is, what do grid cells really do outside of the box. Are they purely spatial? Are they even a grid? What do they do during long scale navigation? How do they represent space when the animal is using that space to perform different behaviors?

II.
What is the adaptive role prefrontal of cortex? We know that animals have progressively evolved larger and larger frontal cortices (Smaers, Gómez-Robles, Parks, & Sherwood, 2017). But at the same time, we know that in a

lab rodents without a cortex can survive while performing and learning multiple behaviors (Kawai et al., 2015; Lopes, Nogueira, Paton, & Kampff, 2016; Whishaw, 1990). They can do many behaviors without involving cortex at all.

On the other hand, studies of the prefrontal cortex in the lab have observed it's activity mostly under tighly controlled and ethologically sterile behavioral paradigms. Over-trained animals doing simple tasks have resulted in observed selective acticity by the mPFC (Kobak et al., 2016).

However, a big question remains: when is prefrontal cortex actively engaged in the natural, survival driven, life of an animal? How come it was evolutionarily selected for? It's critical to study frontal cortex, which we believe is less specialized, and has been strongly selected for in great apes in the context of evolution and ethology (Smaers et al., 2017). We believe it is involved in planning, decision making, etc, but to be fair that's what we have forced the animal to do, sometimes by giving animals too few life choices. It is somewhat *Vox populi* that one can find correlates of anything in mPFC, and by anything it generally means; the controlled elements of one's experiment. However, what does it really do, when the animal is not forced to do anything? Our recent study into the neural activity of mPFC during Hide & Seek shows that even in an unconditioned, highly free behavior, the activity in the mPFC correlates strongly with the structure the behavior takes, and potentially distinguishes between different roles in the same game. This leads us to hypothesize that prefrontal cortex has an important role in modelling the animals role in interaction with other agents in it's environment.

How does this activity relate to the survival or reproduction of the animal? One could answer these question by recording in medial frontal cortices of a ground dwelling rodent in the wild, or in a highly ecological context. Using a reverse physiology approach, we could look at when cortex is active in order to find which natural behaviors seem to be strongly correlating with such activity. Complimentary, performing clustering analysis of the behavior we could reconstruct an ethogram of the animal and identify behavioral clusters and transition points between them. In a way, we would be using the animal's natural stereotypical behavioral repertoire as way of identifying multiple natural experiments (Gomez-Marin, Kampf, Costa, 2014)) and dissecting the role of prefrontal cortex.

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-586!

My number is up.

Juan Ignacio (Nacho) Sanguinetti Scheck
LABO, Berlin, December 2018

Selbständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.

Berlin, den

Juan Ignacio Sanguinetti Scheck